Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions

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The evolution of novelty in tightly integrated biological systems, such as hormones and their receptors, seems to challenge the theory of natural selection: it has not been clear how a new function for any one part (such as a ligand) can be selected for unless the other members of the system (e.g., a receptor) are already present. Here I show—based on identification and phylogenetic analysis of steroid receptors in basal vertebrates and reconstruction of the sequences and functional attributes of ancestral proteins—that the first steroid receptor was an estrogen receptor, followed by a progesterone receptor. Genome mapping and phylogenetic analyses indicate that the full complement of mammalian steroid receptors evolved from these ancient receptors by two large-scale genome expansions, one before the advent of jawed vertebrates and one after. Specific regulation of physiological processes by androgens and corticoids are relatively recent innovations that emerged after these duplications. These findings support a model of ligand exploitation in which the terminal ligand in a biosynthetic pathway is the first for which a receptor evolves; selection for this hormone also selects for the synthesis of intermediates despite the absence of receptors, and duplicated receptors then evolve affinity for these substances. In this way, novel hormone-receptor pairs are created, and an integrated system of increasing complexity elaborated. This model suggests that ligands for some "orphan" receptors may be found among intermediates in the synthesis of ligands for phylogenetically related receptors.

According to the neodarwinian theory of evolution, novel functions arise as the phenotypic outcome of natural selection acting on random mutations. Complex organs and functions are thought to be the result of a gradual selective process of elaboration and optimization (1). Tightly integrated systems of interacting parts, such as those that characterize much of metazoan biology at the molecular level, pose an apparent challenge to this theory, because it is not clear how a new function for any protein can be selected for unless the other members of the complex are already present (2).

Vertebrate steroid hormones and the intracellular protein receptors that mediate their cellular effects elegantly illustrate this problem. In the absence of a ligand, what function does a new receptor serve? And without a receptor, what selection pressures guide the evolution of a new ligand? The six related steroid receptors in vertebrates—the estrogen receptors alpha and beta (ERα and ERβ), progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR)—were created by a series of duplications from a common ancestral receptor gene (3). The classic model suggests that duplicated genes rapidly become pseudogenes unless they are subject to unique selection pressures (4). In theory, new receptors may evolve simultaneously with new ligands (5), or gene duplications may allow multifunctional proteins to take on greater specificity (6).

The history of steroid receptor diversification remains largely unknown. No steroid receptors have been found in any species outside the vertebrates, although an ortholog of the estrogen-related receptor (ERR), the nuclear receptor most closely related to the steroid receptors (3), is present in the *Drosophila melanogaster* genome (Genpept sequence 2891028). (Orthologs are related genes in different genomes, descended from a speciation event; paralogs are related genes in the same genome, descended from a gene duplication.) Orthologs of all steroid receptors present in tetrapods have been identified in teleosts (7, 8), indicating that all six types existed by the time ray-finned fish split from the lineage leading to tetrapods some 400 million years ago (9). PCR screens have identified short fragments of an ER, GR, and AR in shark and a single steroid receptor of indeterminate type in hagfish (8), but this approach cannot distinguish a failure to amplify a gene from its true absence in an organism. I have therefore identified steroid receptors in the sea lamprey *Petromyzon marinus*, which diverged from the jawed vertebrates (gnathostomes) about 450 million years ago (9). Because other gene families contain fewer members in the lamprey than in gnathostomes (10), I anticipated that lamprey would contain a relatively ancient subset of steroid receptors. I used an extensively parallel PCR screen to identify steroid receptor sequences and a phylogenetic approach to determine whether all steroid receptors orthologous to those in extant vertebrates had been obtained. The sequences and functional characteristics of ancestral receptor proteins were reconstructed to illuminate the timing and mechanisms by which the steroid receptor family achieved its current diversity.

Methods

Molecular Methods. Total RNA was extracted from the liver of adult sea lampreys with RNA-zol (Tru-tetst) and reverse transcribed (Superscript from GIBCO). An *Eco*RI-digested cDNA library was prepared in lambda-ZAPII (Stratagene). Degenerate PCR was conducted with ramped temperature profiles (11). For each receptor, at least ten degenerate primers (five in each direction) were used in nested PCR in up to all possible combinations; primers and temperatures are available on request. Products were cloned into pCR2.1 (Invitrogen) and sequenced automatically in both directions. To obtain the entire DNA-binding domain, hinge, and ligand-binding domain, the RACE (rapid amplification of cDNA ends) technique (12) was modified for use on a cDNA library, using nested gene-specific primers that anneal to degenerate PCR products and universal primers that anneal to sequences in the phage.

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Abbreviations: ER, estrogen receptor; AR, androgen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; AncSR, ancestral steroid receptor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY028456, AY028457, and AY028458).

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Sequences, Alignment, and Phylogenetic Analysis. Amino acid sequences of the DNA- and ligand-binding domains of lamprey receptors were inferred and aligned to those of 70 other publicly available steroid and related receptors (see Table 2, which is published as supplemental data on the PNAS web site, www.pnas.org). An elision alignment (13) was prepared in CLUSTALX (14) with gap:change costs in the series 1, 2, 4, . . . , 32 by using the Gonnett weight model. Results that failed to align the AF-2 activation function, which is conserved among all nuclear receptors, were discarded; the remaining alignments (costs 2 to 32) were assembled into a master data matrix. Phylogenetic analyses using parsimony were conducted in PAUP* (15) by using heuristic strategies of multiple random addition and tree bisection–reconnection. A stepmatrix was prepared from the Gonnett model of amino acid transformation (16) by setting diagonal elements to zero and all other elements to the probability of each replacement type if a replacement occurred, then correcting for triangle inequalities. To find the most parsimonious gene family phylogeny, tree lengths were calculated as $L = A + wD$, where A is the number of amino acid replacements, D the number of gene duplications and losses in the reconciled tree (17), and w the weight of a gene duplication/loss relative to a replacement (18). An initial and conservative value of 10 was chosen for w, because duplications and losses of entire genes are expected to occur much less frequently than amino acid changes; the impact of higher and lower values of w was explored analytically. Analyses were conducted without constraint and with multiple topological constraints that limited searches to all possible trees that require fewer gene duplications/losses than the most parsimonious unconstrained tree. Tree lengths, branch lengths, and branch supports were normalized by the average cost of an amino acid change, calculated by dividing the length of the most parsimonious tree with the stepmatrix in effect to the length of that same tree when characters were treated as unordered. Trees were rooted on three nuclear receptor subfamilies closely related to the steroid receptors (3). Confidence in individual nodes was calculated as branch support values—the number of extra steps required in the most parsimonious tree in which that clade does not appear (19)—by using AUTO-DECAY software (20).

Reconstruction of Ancestral Sequences, Functions, and Branch Lengths. Maximum likelihood sequences of ancestral receptors and branch lengths were reconstructed on the most parsimonious phylogeny with PAML software (21)—using a single alignment of 45 steroid receptor sequences for computational efficiency—the Jones amino acid transformation model, and an iteratively estimated gamma distribution of rates ($\alpha = 0.74652$, four categories). Aspects of the ligand specificity of extant receptors were coded as characters and reconstructed for ancestral receptors on the same tree by the parsimony method (22). Ratios of relative rates of sequence divergence were calculated from Poisson-corrected amino acid distances, based on the mean distances of all pairs of ingroup and outgroup sequences (23). The departure of the ratio of means from unity was evaluated by a two-sample t test assuming unequal variances. Teleost receptors were excluded from relative rate tests because of possible rate anomalies after an additional genome-wide duplication (24).

Identification of Paralogous Groups Syntenous with Steroid Receptors. From the OMIM (On-line Mendelian Inheritance in Man) database (http://www.ncbi.nlm.nih.gov), the chromosomal locations of human AR, PR, GR, and MR were ascertained, and the list of genes mapped to the same chromosomes was evaluated for other potential groups of tetralogous genes (25), based on similarity of name among genes shared on two or more of the same chromosomes. Paralogy was verified by BLAST searches of all available human protein sequences (critical value $E < 0.001$) (26), and families with greater than eight members in the human genome were excluded.

Table 1. Pairwise similarities among extant and reconstructed steroid receptor sequences

	humERα	humERβ	humPR	humGR	humMR	humAR
Lamprey ER	63	60	30	32	30	30
Lamprey PR	29	30	66	58	65	59
Lamprey CR	33	32	60	58	60	58
AncSR1	71	68	35	36	33	33
AncSR2	32	30	77	68	72	67

The percent of identical amino acid sites in the combined DNA- and ligand-binding domains is shown for each pair.

Results and Discussion

Lampreys Contain an Ancient Subset of Steroid Receptors. An extensively parallel PCR strategy yielded lamprey steroid receptors when primers designed from gnathostome PR, ERα, and GR were used. The first receptor was most similar to the gnathostome PR and was named lamprey PR; the second was similar to gnathostome ERα and ERβ and was named lamprey ER; the third was similar to both of the vertebrate corticoid receptors MR and GR and was named lamprey CR (Table 1). For those receptors that were not amplified, reactions were repeated under varying conditions, and additional primers were designed when possible. Despite more than 200 unique degenerate PCR reactions, no authentic steroid receptor sequences were amplified for the ERβ, AR, or MR.

A PCR screen cannot exclude the possibility that orthologs to these receptors exist in the lamprey genome but were not recovered because of a lack of expression or extreme sequence divergence. Gene family phylogenetic analysis, however, can determine the timing of gene duplication events relative to speciation events and thereby offers a powerful method to distinguish a false negative PCR result from a real lack of these receptors in any taxon (27). The most parsimonious phylogeny of the steroid receptor gene family was inferred based on the sequences of the three lamprey receptors and 70 other publicly available sequences. Any gene family tree implies a certain number of gene duplications and losses, and genealogical inference should take account of both sequence evolution and changes in the presence/absence of a gene. In a phylogenetic context, the most parsimonious and therefore best supported tree is the one that minimizes the sum of weighted amino acid replacements and gene duplications/losses (18). When using this approach and a conservative cost ratio for duplications/losses to amino acid replacements, the single most parsimonious tree of the steroid receptors (Fig. 1) implies no extra duplications and losses beyond the minimum required to explain the distribution of receptors in lamprey and gnathostomes. This phylogeny is the most parsimonious for any and all cost ratios greater than 3, an implausibly low value, because amino acid replacements are almost certainly more than three times as likely as duplication or loss of an entire gene. The tree is well supported, with 3 to 24 extra amino acid changes required to impose alternative relationships at any of the nodes relevant to this analysis, indicating that the sequence data's support for this phylogeny is unlikely to be due to chance effects or phylogenetic noise.

The steroid receptor phylogeny indicates that unique orthologs to the AR, MR, and ERβ were not recovered from the lamprey because these receptors were created by gene duplication in the jawed vertebrate lineage, after the lamprey–gnathostome divergence. If the AR gene, for example, had appeared by duplication before this cladogenetic event, then the lamprey PR would form a clade with the gnathostome PRs to the

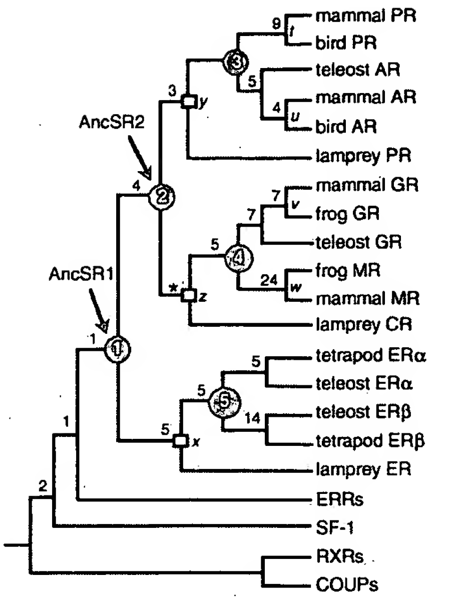


Fig. 1. Phylogeny of the steroid receptor gene family. A reduced version is shown of the single most parsimonious phylogeny of 73 receptor sequences when the relative weight of gene duplications/losses to amino acids $w > 3$. (Length = 3,209 aa changes + 8 duplications + 0 losses. For unreduced phylogeny, see Fig. 7, which is published as supplemental data.) Support for each clade is shown as the number of extra steps required for the labeled node not to appear in the most parsimonious tree (19); all support values are insensitive to w except *, shown for w = 10. Blue circles indicate gene duplications within the steroid receptor family; red squares mark the lamprey–gnathostome divergence; and unmarked nodes represent other speciation events. Ancestral steroid receptors are indicated. Italicized node labels correspond to Fig. 3. Tree length = 3,209 aa changes, eight duplications, zero losses; consistency index = 0.628; retention index = 0.870.

Chromosome 4	5	11	X
MR	GR	PR	AR
		API2	API3
ART3		ART1	
	CDX1		CDX4
	CTNND2	CTNND1	
	DIAPH1		DIAPH2
		DLG2	DLG3
DRD1B	DRD1		
FABP2	FABP6		
FACL2			FACL4
FGFR3	FGFR4		
GLRA3	GLRA1		GLRA2
GRIA2	GRIA1	GRIA4	GRIA3
	HSPL27	HSP27b2	
IRF2	IRF1		
LGMD2E	LGMD2F		
MADH1	MADH5		
		MTMR2	MTMR1
NFKB1		NFKB3	
NPY2R	NPY6R		
PDGFRA	PDGFRB		
PDHA2			PDHA1
PKD2	PDK2L2		
PPEF2			PPEF1
PPP3CA	PPP2C1	PPP1CA	PPP6C
PTTG2	PTTG1		
		RDX	MSN
SNCA	SNCB		
	SYT4	SYT5	
TXK	ITK		BTK

Fig. 2. Steroid receptors diversified by large-scale genome expansions. Paralogous members of gene families with two or more members on the same chromosomes as the human 3-ketosteroid receptors are shown, without regard to map order.

exclusion of the gnathostome ARs; the phylogeny obtained here, however, shows that the lamprey–gnathostome divergence occurred before the gene duplication that created the AR. By similar reasoning, lampreys must possess one estrogen receptor ancestral to the gnathostome ERα and ERβ, and one corticoid receptor ancestral to the GR and MR. This analysis does not rule out the possibility of independent gene duplications in the lamprey lineage that may have created other novel receptor paralogs, but it does indicate that the three sequences recovered represent the entire complement of steroid receptors orthologous to the six found in jawed vertebrates.

Steroid Receptors Diversified in Two Serial Genome Expansions. The receptor phylogeny suggests that two serial duplications of an ancestral steroid receptor occurred before the divergence of lamprey and jawed vertebrates. The first created an estrogen receptor and a 3-ketosteroid receptor, whereas the second duplicated the latter gene to produce a corticoid receptor and a receptor for 3-ketogonadal steroids (androgens, progestins, or both). The ancestral vertebrate therefore had three steroid receptors—an estrogen receptor, a receptor for corticoids, and a receptor that bound androgens, progestins, or both. At some later time within the gnathostome lineage, each of these three receptors duplicated yet again to yield the six steroid receptors currently found in jawed vertebrates: the ER to create ERα and ERβ, the corticosteroid receptor to yield the GR and the MR, and the 3-ketogonadal steroid receptor to create the PR and the AR.

This finding is consistent with the hypothesis that the genome of "higher" vertebrates is the result of two genome duplication events that occurred early in chordate evolution (24, 28). The timing of these events has remained unclear, however, because the number of members of other gene families in the lamprey has been consistent with two competing hypotheses: two duplications before the lamprey–gnathostome divergence, or one duplication before and one after (29). The number of steroid receptors in lamprey and their phylogenetic relations to gnathostome sequences clearly support the latter hypothesis.

Gene mapping data also support serial genome duplications as the mechanism by which steroid receptors diversified. When a gene family is created by large-scale duplications rather than by local processes like tandem duplication or transposition, its members will be syntenous—mapped to the same chromosome—with members of other gene families that proliferated in the same events. Redundant genes will often be lost after gene duplications; therefore, many of the paralogous groups that result from two rounds of gene duplication are expected to contain two or three rather than four members (25). The complete human genome sequence suggests that large-scale block duplications have occurred, but mapping data from a single species are not enough to distinguish whole-genome duplications from regional copying of chromosomes or their parts (30). Mapping data from the human genome (Fig. 2) indicate that the AR, PR, GR, and MR on chromosomes X, 4, 5, and 11 are syntenous with members of at least 30 other gene families. The actual number of syntenous gene families is likely to be considerably higher, because the identification criteria used were conservative. The large number of linked paralogous groups indicates that the steroid receptors diversified as the result of two rounds of large-scale genome expansion rather than by gene-specific mechanisms like transposition or tandem duplication. Complete genome sequences from lamprey and other gnathostomes, along with development of numerical models of the chromosomal distribution of genes, are required for statistical testing of this hypothesis.

The Ancestral Steroid Receptor Was an Estrogen Receptor. The classical model of gene duplication suggests that redundancy will

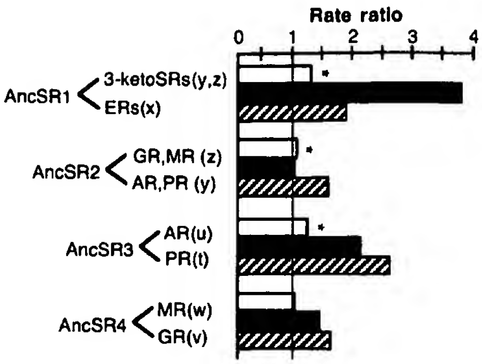


Fig. 3. Divergence rates of steroid receptor sequences after gene duplications indicate that the first steroid receptor was an estrogen receptor. Grouped bars show the ratio of the rate of amino acid replacement on the upper branch to that in the lower. White bars, rate ratio based on the relative rate test; black bars, ratio of parsimony branch lengths; hatched bars, ratio of maximum likelihood branch lengths. Outgroups for relative rate tests, from top to bottom, are estrogen-related receptors (ERRs), tetrapod ERs, lamprey PR, and lamprey GR. *, statistically significant departure from unity, $P < 0.001$. Parsimony and likelihood branch lengths are proportional to the number of weighted amino acid changes on paralogous branches that descend from duplication of an ancestral steroid receptor to an equivalent speciation event, with labels corresponding to nodes in Fig. 1.

a)

	Anc SR1	hum ERα	Anc SR2	hum PR	hum GR	hum MR	hum AR	# in humERa	# in humPR
1	R	○	○	○	○	○	○	394	766
2	E	○	Q	·	·	·	·	353	725
3	L	●	M	·	·	S	·	387	759
4	L	●	●	●	V	M	●	354	(726)
5	L	●	●	●	●	●	●	349	721
6	A	●	C	G	G	A	G	350	(722)
7	T	●	N	·	·	·	·	347	719
8	L	●	●	●	●	●	●	346	718
9	L	●	M	·	●	●	·	540	909
10	L	M	●	●	M	●	●	343	715
11	M	●	●	F	●	●	●	421	(794)
12	V	●	I	●	·	●	●	534	(903)
13	M	●	T	·	·	·	L	528	894
14	L	●	C	·	·	·	T	525	891
15	L	●	F	·	·	·	·	(536)	905
16	H	○	F	Y	Y	·	·	524	890
17	A	G	L	·	·	·	·	521	887
18	M	●	L	H	·	·	H	522	(888)
19	F	●	C	·	·	·	·	425	(798)
20	L	I	●	●	Q	●	Q	424	797
21	L	●	M	·	·	·	·	428	801
22	M	●	A	V	·	S	V	388	(760)
23	W	●	●	●	●	●	●	383	755
24	M	L	●	●	●	●	●	384	756
25	L	●	M	●	●	●	·	391	(763)
26	F	●	●	●	●	●	●	404	778
27	L	●	●	●	●	●	●	402	(776)

b)

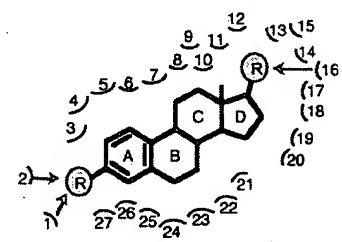
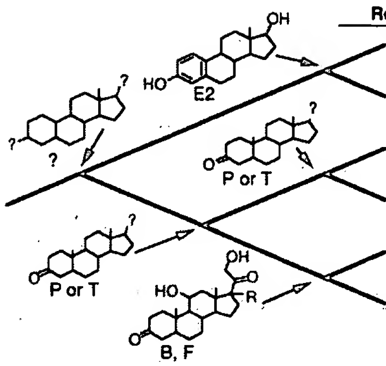


Fig. 4. Maximum likelihood reconstructions of ancestral sequences indicate that the first steroid receptor was an estrogen receptor and the first 3-ketosteroid receptor was not an androgen receptor. (*a*) Aligned amino acids forming the ligand-binding pocket of the ancestral steroid receptor (AncSR1), the ancestral 3-ketosteroid receptor (AncSR2), and five human steroid receptors, based on homology to human steroid receptors with solved structures. Colors and residue numbers refer to the positions shown in *b*. Filled circles (●) indicate amino acids identical to AncSR1; small dots are identical to AncSR2 but not AncSR1. Red, amino acids making direct hydrogen bonds with ligands (32, 33); blue, residues critical to discriminate androgens from C21 steroids in the androgen receptor (35). Amino acid numbers of homologous positions in the crystallized human receptors are at right; parentheses indicate positions that do not contact ligand in the indicated receptor. (*b*) Schematic of the ligand-binding pocket of ancestral steroid receptors with generic steroid hormone, based on homology to the crystal structures of human ERα and human PR (32, 33). Red and blue residues as in a. Yellow circles marked "R" indicate substituents that vary among steroid hormones.

free one member of the resulting pair of genes from the constraints imposed by natural selection, and its sequence will diverge rapidly, yielding either a pseudogene or, less commonly, a gene with novel functions, the sequence of which will then be constrained again by selection (4). An ancestral protein is likely to have been most similar in sequence and therefore in function to the descendant gene that diverged more slowly after the duplication event than the one with a more rapid evolutionary rate. Relative rate tests based on amino acid distances and reconstruction of branch lengths by parsimony and maximum likelihood all indicate that the rate of amino acid replacement after duplication of the ancestral steroid receptor (AncSR1) was up to four times greater in the lineage leading to the 3-ketosteroid receptors than in that leading to the estrogen receptors, a result that is statistically significant at a $P = 0.001$ level in the relative rate test (Fig. 3). The gross difference in sequence divergence rates suggests that that the ancestral steroid receptor was a functional estrogen receptor, the sequence of which was conserved among descendant ERs.

The amino acid sequence of the ancestral steroid receptor was reconstructed with a maximum likelihood approach; this ancestral sequence, interpreted in light of structure-function data on extant vertebrate receptors, strongly support the inference that AncSR1 was an estrogen receptor. The reconstructed ancestral receptor is 71% identical to the human ERα, but radically less similar to the PR, AR, GR, and MR (Table 1). In its DNA-binding domain, the ancestral receptor shares 61 of 66 residues with the human ERα, but no more than 41 with any of the nonestrogenic receptors (see Fig. 6, which is published as supplemental data). The ancestral sequence's P-box, the sequence of which determines the distinct specificity of the ER and the GR for their respective target sequences (31), has the exact sequence of estrogen receptors rather than that found in the AR, PR, GR, and MR (Fig. 6). This result indicates that the ancestral steroid receptor activated genes with estrogen-response elements (a palindrome of AGGTCA) rather than those with the response elements recognized by the other steroid receptors (a palindrome of AGAACA). These data strongly suggest that ancestral steroid receptor bound estrogens and activated genes regulated by classic estrogen-response elements.

Reconstructions for specific amino acid positions that contact hormone in the crystallized human ERα provide more specific support (Fig. 4). Of 20 positions that vary among steroid receptors, 16 contain the residue characteristic of the estrogen receptor, and all four exceptions are conservative replacements. Most tellingly, twelve of the residues that AncSR1 shares with the ER are diagnostic of estrogen receptors in that all other steroid receptors have different amino acids at these positions. The reconstructed ancestral receptor has the ER-characteristic residue at the critical position that forms hydrogen bonds with and discriminates between the 3-hydroxyl group of estrogens and the 3-keto group of all other steroid hormones, as well as at the position that bonds with and discriminates the 17-hydroxyl group of estrogens from the methylketo moiety of progesterone and corticoids (32, 33). At seven of the eight positions that surround



Receptor	Ligand	3-	11-	17β-	21-
ERα	E2	-OH	-H	-OH	NA
ERβ	E2	-OH	-H	-OH	NA
PR	P	=O	-H	-COCR	-H
AR	T	=O	-H	-OH	NA
GR	B, F	=O	-OH	-COCR	-OH
MR	B, F, Aldo	=O	-OH	-COCR	-OH

Fig. 5. Reconstruction of ligand-binding characteristics of ancestral steroid receptors indicates that the ancestral 3-ketosteroid receptor did not bind corticoids. Substituents at critical positions of the ligands that each vertebrate receptor binds were coded as characters (*Right*). Character states at ancestral nodes were reconstructed on the reduced phylogeny of steroid receptor paralogs with a parsimony-based algorithm, and the inferred structures of the ligands bound by each ancestral receptor are shown (*Left*); colored groups correspond to characters in the matrix. ?, substituent groups that could not be unambiguously reconstructed; NA, not applicable. P, progesterone; E2, estradiol; T, testosterone; B, corticosterone; F, cortisol; Aldo, aldosterone.

the steroid D-ring, which varies among estrogen, progesterone, and corticoids, the ancestral receptor's amino acid is identical to the ER but different from the other receptors. If the estrogen receptor is the most ancient of all steroid receptors, it is likely also to have the widest taxonomic distribution, suggesting that a broad array of taxa may be potentially sensitive to synthetic environment pollutants that interact with the estrogen receptor (34).

The Second Receptor to Evolve Was a Progesterone Receptor. Maximum likelihood sequence reconstructions indicate strongly that the ancestral 3-ketosteroid receptor (AncSR2) bound C21 steroids—progestins or corticoids—and not C19 androgens, and they suggest that this protein was a progesterone receptor. The combined DNA- and ligand-binding domains are 81% identical to the human PR, but considerably less similar to the AR, GR, and MR (Table 1). Of the amino acids that form the ligand-binding pocket in the human PR and are not shared with the AR, GR, and MR, the ancestor shares more residues with the PR—and fewer with the AR—than with any other extant receptor (Fig. 4). Positions that align with C891 and T894 in the human PR are critical, because crystallographic studies show that these amino acids contact the C20 keto group unique to progesterone and corticoids (33), and mutation of the former residue in the human AR to that found in the other receptors causes the AR to bind and trans-activate in the presence of progesterone and corticoids (35). At both of these positions, the reconstructed ancestor contains residues characteristic of the PR, GR, and MR, but not AR, indicating that it bound C21 steroids rather than C19 androgens like testosterone or dihydrotesterone.

Branch length comparisons support this inference. By all methods of calculation, the rate of sequence divergence after the duplication of the common ancestor of the androgen and progesterone receptors (AncSR3) is considerably greater in the lineage leading to the AR than in that leading to the PR, and the difference is statistically significant in the relative rate tests (Fig. 3). This result suggests that the androgen receptor is a more recent evolutionary novelty than the progesterone receptor.

Reconstruction of the ability of ancestral receptors to bind specific ligands by using a parsimony-based algorithm, which explains shared states as due to descent from a common ancestor, indicates that the ancestral 3-ketosteroid receptor was a PR (Fig. 5). This reconstruction shows that AncSR2 did not bind corticoids; the capacity to bind 11- and 21-hydroxylated steroids evolved later on the branch leading to the GR and the MR. If the AR was a late evolutionary novelty—as both branch lengths and sequence reconstructions show—and if the ancestral 3-ketosteroid receptor bound progestins or androgens but not corticoids, then the second receptor to evolve would have been a PR.

Ligand Exploitation: A Mechanism for the Evolution of Endocrine Complexity. These findings support a model for hormone/receptor evolution in the steroid receptor family, the dynamics of which may also apply to other kinds of receptors and their ligands. If, as I have shown, the first receptor to evolve was an estrogen receptor, then the terminal hormone in the pathway for steroid biosynthesis was the first to function as a hormone acting through an intracellular receptor. In the synthesis of estradiol and other estrogens, progesterone and testosterone are synthesized as intermediates. These steroids (and the enzymes that produce them) would therefore have been present during the period when only a receptor for estrogen existed. After one to three duplications of the estrogen receptor gene, followed by considerable sequence divergence, receptors emerged that gave these intermediate compounds novel signaling functions. The advent of corticoid signaling would have required enzymes for 21- and 18-hydroxylation to be added to the pathway.

This evolutionary history provides one solution to the problem posed by the classical model—how can a ligand or a receptor be maintained without the other in a system governed by natural selection? Once an organism depended on estrogen/ER signaling for physiological or developmental functions that contribute to fitness, then the same constraints that selected for the synthesis of estrogen and its receptor would by necessity have selected for the synthesis of other steroids in the pathway, although none of them yet signaled through nuclear receptors. Redundant receptors created by gene duplication could then diverge in sequence from their ancestors and evolve affinity for these steroids, creating signaling functions for what were once intermediates. I call this process ligand exploitation, because it involves the cooption of existing metabolites to serve as novel hormones by duplicated receptors; this model reverses the evolutionary dynamics previously proposed for hormone-receptor evolution (36). Ligand exploitation can occur whether ancestral receptors regulated cellular processes through direct transcriptional activation, via signal transduction pathways in the cytosol or membrane, or both, as extant steroid receptors do (37, 38). If ligand exploitation is a general mechanism for the evolution of new receptor-hormone pairs, then ligands for some

"orphan" receptors may be found among intermediates formed in the synthesis of ligands for phylogenetically related receptors.

Evolution of Endocrine Specificity. The elaboration of the steroid receptor family by gene duplication and ligand exploitation allowed increasingly specific hormonal control over physiological functions. Estrogen regulation, presumably of reproductive maturation and function, appears to be the most ancient of all modes of steroid/receptor control, a conclusion supported by the apparent role of estrogen in branchiostome and echinoderm reproduction (39, 40). Progesterone control over ovulation, oviposition, or other aspects of reproduction also appears to be quite ancient, as indicated by the presence of corpora atretica and lutea in hagfish ovaries (41), but not as old as estrogen signaling. Hormonal control over sexual dimorphism appears to be a relatively recent evolutionary novelty: if the androgen receptor was created by a gene duplication after the lamprey lineage diverged from other vertebrates, then androgen-mediated masculinization and estrogen-mediated feminization must be unique to the gnathostomes. Supporting this view, the lamprey testis binds estradiol but not androgens with high affinity (42); in both male and female lamprey, estradiol regulates reproductive maturation and behavior, but androgens do not appear to play any role, and plasma levels of neither hormone are sexually dimorphic (43, 44).

Separate control over osmolarity and response to stress must also have arisen after the lamprey–gnathostome divergence. In many jawed vertebrates, including mammals, osmolarity is regulated by the MR, whereas the GR controls long-term stress response (45). Lampreys, with a single CR, would be expected to have no ability to use steroids for independent regulation of these functions. Corticoids are found in plasma and appear to regulate osmolarity in lamprey and hagfish, but no research on their glucocorticoid effects is available (46, 47). The spawning behavior of most lamprey species, however, involves a migration from marine to freshwater environments accompanied by extreme and fatal changes in carbohydrate and protein metabolism, consistent with coordinate control of these functions. Independent regulation of the many physiological functions controlled by steroids in jawed vertebrates therefore appears to have been gradually elaborated from an ancient mechanism for estrogen regulation, as receptor genes duplicated, diverged, and exploited the middle steps of a biosynthetic pathway that was stabilized by natural selection acting on its endpoint.

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Acceleration of granulocyte colony-stimulating factor-induced neutrophilic nuclear lobulation by overexpression of Lyn tyrosine kinase

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Stimulation with granulocyte colony-stimulating factor (G-CSF) induces myeloid precursor cells to differentiate into neutrophils, and tyrosine phosphorylation of certain cellular proteins is crucial to this process. However, the signaling pathways for neutrophil differentiation are still obscure. As the Src-like tyrosine kinase, Lyn, has been reported to play a role in G-CSF-induced proliferation in avian lymphoid cells, we examined its involvement in G-CSF-induced signal transduction in mammalian cells. Expression plasmids for wild-type Lyn (Lyn) and kinase-negative Lyn (LynKN) were introduced into a murine granulocyte precursor cell line, GM-I62M, that can respond to G-CSF with neutrophil differentiation, and cell lines that overexpressed these molecules (GM-Lyn, GM-LynKN) were established. Upon G-CSF stimulation, both the GM-Lyn and GM-LynKN cells began to differentiate into neutrophils, showing early morphological changes within a few days, much more rapidly than did the parental cells, which started to exhibit nuclear lobulation about 10 days after the cells were transferred to G-CSF-containing medium. However, the time course of expression of the myeloperoxidase gene, another neutrophil differentiation marker, was not affected by the overexpression of Lyn or LynKN. Therefore, in normal cells, protein interactions with Lyn, but not its kinase activity, are important for the induction of G-CSF-induced neutrophilic nuclear lobulation in mammalian granulopoiesis.

Keywords: differentiation; granulocyte colony-stimulating factor (G-CSF); granulocyte; lobulation; neutrophil.

The production of blood cells is regulated by a variety of extracellular stimuli, including a network of hematopoietic growth factors and cytokines [1]. Among them, granulocyte colony-stimulating factor (G-CSF) is a critical regulator of neutrophilic granulocyte production and stimulates the proliferation, survival, maturation, and functional activation of the cells of the granulocytic lineage [2,3]. A variety of G-CSF activities are mediated through its interaction with a specific cell-surface receptor [3,4]. Molecular cloning of the G-CSF receptor cDNA revealed that it is a type I membrane protein consisting of about 800 amino acids and that it belongs to the hematopoietic growth factor receptor family [5,6]. On ligand binding, the G-CSF receptor forms a homodimer, which induces the signal transduction [7]. Like other members of the cytokine receptor superfamily, the G-CSF receptor has no intrinsic tyrosine kinase activity, but activates cytoplasmic tyrosine kinases. Signaling molecules reported to be activated through the G-CSF receptor include the Janus tyrosine kinases Jak1, Jak2, and Tyk2 [8–11], the signal transducer and activator of transcription (STAT) proteins STAT1, STAT3, and STAT5 [8,12–14], the Src kinases Lyn and Hck [15–17], and components of the Ras, Raf, mitogen-activated protein kinase (MAPK) and MAPK-related pathways [18–22].

The cytoplasmic region of the G-CSF receptor can be subdivided into a membrane-proximal domain, which has two conserved subdomains designated box 1 and box 2, and a membrane-distal domain, which has four tyrosine residues at positions 703, 728, 743, and 763 of the murine receptor. The membrane-proximal domain is known to be a binding site for the Jak family of tyrosine kinases and is essential for mitogenic signaling, whereas both the membrane-proximal domain and the membrane-distal domain are indispensable for the transduction of differentiation signals [23,24]. Binding of G-CSF to its receptor results in the rapid phosphorylation of these four tyrosine residues in the cytoplasmic domain [25,26], which form potential binding sites for signaling molecules that contain Src homology 2 (SH2) or phosphotyrosine-binding domains [27]. Indeed, the first (Tyr703) and the third (Tyr743) tyrosines from the membrane-spanning domain have been reported to be the STAT3-docking sites when these residues are phosphorylated [14,28–31]. In addition, the fourth (Tyr763) tyrosine is necessary for the G-CSF-dependent phosphorylation of Shc and the activation of the $p21^{ras}$-MAPK signaling pathway [21,32].

Besides the Jak family of kinases, G-CSF stimulation induces the activation of nonreceptor protein tyrosine kinases, such as the Src-like kinase Lyn and the tandem

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Abbreviations: G-CSF, granulocyte colony-stimulating factor; IL-3, interleukin-3; MAPK, mitogen-activated protein kinase; MPO, myeloperoxidase; STAT, signal transducer and activator of transcription; SH2, Src homology 2; SH3, Src homology 3; HRP, horseradish peroxidase; EF-1α, elongation factor-1α.

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SH2-containing kinase Syk [16]. These tyrosine kinases have been reported to be associated with the G-CSF receptor, but their physiological roles are not clearly understood. In avian hematopoietic Lyn-deficient cells, ectopic expression of the human G-CSF receptor failed to reconstitute G-CSF-dependent mitotic responses, leading to the conclusion that Lyn is required for G-CSF-induced DNA synthesis [17].

To investigate the role of Lyn kinase in the G-CSF-induced signaling pathway in mammalian hematopoietic cells, we overexpressed wild-type Lyn (Lyn) and kinase-negative Lyn (LynKN) in murine granulocyte progenitor cells GM-I62M and examined their G-CSF responses. We found that cells that overexpressed either form of Lyn responded to G-CSF with morphological changes, including nuclear lobulation, much more rapidly than did the control cells. Therefore, protein–protein interactions with Lyn, but not its kinase activity, appear to regulate G-CSF-induced nuclear lobulation.

MATERIALS AND METHODS

Factors and cell lines

Mouse recombinant interleukin-3 (IL-3) and G-CSF were as described previously [33]. Their biological activities were determined by measuring their ability to stimulate [^{3}H]thymidine incorporation in the mouse IL-3-dependent myeloid cell line, NSF-60 [34]. One unit of activity represents the concentration of CSF required for the half-maximal stimulation of 5×10^4 cells per 100 μL. The mouse myeloid cell line GM-I62M [26], which is an LGM-1 transformant expressing the mouse G-CSF receptor, was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Gibco BRL, Rockville, MD, USA) and 45 U·mL^{-1} IL-3.

Plasmid construction

The Flag-tagged expression vector was constructed as follows. PCR was carried out using BOS-5 (GGGTTTG CCGCCAGAACACA) and BOS-Flag-rev (CCGAATT CCTTGTCATCGTCATCCTTGTAGTCCATGGTGGC CTCACGACACCTGA) primers with pEF-BOS-EX expression plasmid [35] as the template. The resultant 1-kbp DNA fragment was isolated and digested with *Xho*I and *Eco*RI. The *Xho*I–*Eco*RI region of the pEF-BOS-EX plasmid was replaced with the 150-bp *Xho*I–*Eco*RI PCR fragment. The DNA sequence of the 150-bp *Xho*I–*Eco*RI fragment in the plasmid pEF-BOS-EX-Flag was confirmed by sequencing. pEF-BOS-EX-Flag contains a DNA fragment encoding the Flag peptide just upstream of the *Eco*RI site of the multiple cloning site of pEF-BOS-EX.

Murine Lyn cDNA was isolated by RT-PCR from the total RNA of GM-Y2M cells [26]. cDNA for the N-terminal half of Lyn was amplified with primer Lyn-Nfor (GCGAATTCCGAGCGAGAAATATGGG) and internal primer Lyn-Nrev (AACTGCCCTGCGCCAAGC), while cDNA for C-terminal Lyn was amplified using primers Lyn-Cfor (TCACTTTTCCCTGCATCAG) and Lyn-Crev (GCTCTAGACAATAGGCTAGTCTCC). The resultant DNA fragments were inserted into the *Sma*I site and the *Sma*I, *Xba*I sites of pBluescriptII KS(+) (Stratagene, La Jolla, CA, USA), respectively, and were named pBS-LynN and pBS-LynC. The authenticity of pBS-LynN and pBS-LynC was confirmed by DNA sequence analysis, and these sequences were identical with the corresponding regions of mouse Lyn cDNA (accession number M64608) [36]. Flag-tagged full-length Lyn expression plasmid (pBOS-FlagLyn) was constructed by ligating the *Eco*RI–*Sph*I fragment of pBS-LynN, the *Sph*I–*Xba*I fragment of pBS-LynC, and the *Eco*RI–*Xba*I-digested pEF-BOS-EX-Flag.

To construct the LynKN expression plasmid, site-directed mutagenesis with PCR [37] was carried out to replace Lys275 with Arg at the ATP-binding site of the kinase domain. The primers were M13-reverse (CAG GAAACAGCTATGACCAT) and lyn-KNrev (CTTGAG GGTCCTCACAGCCAC) for one reaction, and lyn-KNfor (GTGGCTGTGAGGACCCTCAAG) and Lyn-Crev (GC TCTAGACAATAGGCTAGTCTCC) for another, with pBS-LynC as the template. Both products were isolated by agarose gel electrophoresis, then mixed 1 : 1 and used as templates for secondary PCR with Lyn-Cfor and Lyn-Crev as primers. The PCR product was digested with *Sph*I and *Eco*RI, and the resultant 611-bp DNA fragment was inserted into pUC18, which had been digested with *Sph*I and *Eco*RI. The authenticity of the plasmid obtained (pUC18-LynC-mt) was confirmed by DNA sequencing. The *Sph*I–*Eco*RI fragment of pUC18-LynC-mt was isolated again and ligated with the *Sph*I–*Bgl*II fragment and the *Bgl*II–*Eco*RI fragment of pBOS-FlagLyn. The plasmid obtained was designated pBOS-FlagLynKN and used as an expression plasmid for Flag-tagged LynKN.

Transfection

Mouse GM-I62M cells were transfected with pBOS-Flag-Lyn or pBOS-FlagLynKN with pBSpacΔp [38], which carries the puromycin-resistance gene, by electroporation (350 V pulse, 250 μF capacitance) using a Gene Pulsar II (Bio-Rad Laboratories, Hercules, CA, USA), essentially as described [39]. In brief, 5×10^6 cells were transfected with 40 μg *Apa*LI-digested pBOS-FlagLyn or pBOS-Flag-LynKN together with 1 μg pBSpacΔp, which had been digested with *Eco*RI. Thereafter, cells were cultured for 24 h and then selected with medium containing puromycin (0.75 μg·mL^{-1}) for 2 weeks. Puromycin-resistant clones were expanded and tested for their expression of the Flag-tagged Lyn or LynKN proteins by immunoblot analysis with an anti-Flag M2 IgG (Sigma, St Louis, MO, USA). Transformants were grown in RPMI 1640 medium containing 10% fetal bovine serum and mouse IL-3 (45 U·mL^{-1}).

Assay of long-term cell growth and morphological examination

To determine the long-term growth potential of the Lyn-expressing transformants, cells were incubated at an initial density of 1×10^5 cells·mL^{-1} in medium containing no factor, 150 U·mL^{-1} mouse G-CSF, or 45 U·mL^{-1} mouse IL-3. The medium was replenished every 2–3 days to maintain the cell density at $(1–5) \times 10^5$ cells·mL^{-1}. Viable cells were counted under the light microscope. To analyze the morphological changes, cells were collected on glass slides by centrifugation (850 g for 5 min at 4 °C) and stained with Wright–Giemsa solutions (E Merck).

Assay of thymidine incorporation

A total of 1.5×10^4 cells in 100 μL RPMI 1640 containing 10% fetal bovine serum and various concentrations of G-CSF or IL-3 were incubated at 37 °C for 22 h. Then 0.5 μCi [^{3}H]thymidine (Amersham Pharmacia Biotech, Inc. Piscataway, NJ, USA) was added and the cells were further incubated for 4 h at 37 °C before being harvested.

Cytokine stimulation and immunoblotting

Cells were grown in the presence of IL-3 to a density of up to 1×10^6 cells·mL^{-1}, washed twice with factor-free medium containing 5% fetal bovine serum, and starved in the factor-free medium with 10% fetal bovine serum at 2×10^6 cells·mL^{-1} for 5 h. After being stimulated with 150 U·mL^{-1} G-CSF for the period indicated for each experiment, cells were immediately chilled on ice/water, washed twice with ice-cold NaCl/P$_i$, and lysed with lysis buffer [50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 0.5% CHAPS, and protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 1 μg·mL^{-1} each leupeptin and pepstatin A; Sigma)] for 15 min on ice at a cell density of 1×10^8 cells·mL^{-1}. Insoluble materials were removed by centrifugation at 14 000 ***g*** for 15 min at 4 °C. Cellular proteins were subjected to SDS/PAGE and blotted on to GVHP membranes (Millipore Corp., Bedford, MA, USA) as described previously [26]. The membranes were incubated with primary antibody [anti-phosphotyrosine IgG (4G10) (Upstate Biotechnology Inc., Lake Placid, NY, USA), anti-Flag M2 IgG (Sigma) or anti-Lyn IgG (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA)] and a rabbit anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Carpinteria, CA, USA) or goat anti-rabbit IgG HRP-conjugated secondary antibody (Bio-Rad, Richmond, CA, USA), then visualized by enhanced chemiluminescence (Renaissance, Dupont NEN, Boston, MA, USA), as described previously [26].

Northern-blot analysis

Cells cultured in the presence of IL-3 were washed twice with factor-free medium containing 5% fetal bovine serum and starved for 4 h in the factor-free medium with 10% fetal bovine serum, then G-CSF (150 U·mL^{-1}) or IL-3 (45 U·mL^{-1}) was added to the medium, and the cells were cultured for another 48 h. Total RNA was extracted from the cells using guanidine isothiocyanate/phenol/chloroform [40]. Northern-blot hybridization was carried out as described previously [23]. As probes, murine myeloperoxidase (MPO) cDNA [41] or human elongation factor-1α (EF-1α) cDNA [42] were labeled with [^{32}P]dCTP (Institute of Isotopes Co., Ltd, Budapest, Hungary) using a random primer DNA labeling kit (Takara, Tokyo, Japan).

RESULTS

Expression of Lyn cDNA in myeloid cell line GM-I62M

To examine the roles played by the protein tyrosine kinase Lyn in the G-CSF signal-transduction pathway, full-length mouse Lyn cDNA was isolated by RT-PCR from total RNA prepared from the GM-Y2M cell line [26]. The cDNA was sequenced and found to be identical with mouse Lyn (GenBank accession number M64608). The cDNA for LynKN was constructed using PCR, replacing Lys275 with Arg. The cDNAs for wild-type Lyn and LynKN were inserted into the mammalian expression plasmid pEF-BOS-EX-Flag, in such a way that the Flag peptide was fused to the N-terminus of the molecule (pBOS-Flag-Lyn, pBOS-Flag-LynKN).

GM-I62M cells proliferate in the presence of IL-3 and respond to G-CSF by undergoing neutrophil maturation. They start expressing MPO mRNA within a few days and show nuclear lobulation about 10 days after being transferred to G-CSF-containing medium. Both the Lyn-expressing and LynKN-expressing plasmids (pBOS-Flag-Lyn, pBOS-Flag-LynKN) were introduced into the GM-I62M cell line along with a puromycin resistance gene, and the resulting cell lines were selected using puromycin resistance. The expression of Lyn and Lyn-KN in the cell lines was confirmed by immunoblotting the cell lysates using an anti-Flag IgG. As shown in Fig. 1A, GM-Lyn and GM-LynKN expressed fairly large amounts of Flag-Lyn and Flag-LynKN, as judged by immunoblot analysis with the anti-Flag IgG, while the parental cell line, GM-I62M, as expected, did not. The quantities of the Lyn proteins in these cell lines were about 10 times that of the endogenous Lyn protein, as estimated by immunoblot analysis with an anti-Lyn IgG (Fig. 1B). These cell lines were used to investigate G-CSF responses in the following experiments. A couple of other cell lines that expressed Lyn or LynKN in similar quantities were also examined and gave the same results (data not shown).

Effects of Lyn expression on the G-CSF-dependent growth and differentiation response

The growth of GM-I62M cells depends on IL-3 and they also respond to G-CSF by proliferating. However, the cells stop dividing after 4–5 days of culture in the presence of G-CSF and start to differentiate into neutrophils (Fig. 2). To examine the effects of Lyn expression on the G-CSF-dependent cell responses, cells overexpressing Lyn or LynKN were starved for 4 h and transferred to medium containing G-CSF. As shown in Fig. 2, cells expressing both Lyn and LynKN proliferated for 4–5 days in the presence of G-CSF. After this time, the cell number stayed constant, as also seen in the parental cell line, GM-I62M. Therefore, G-CSF-dependent growth properties were not affected by the overexpression of Lyn or LynKN.

To test neutrophil differentiation in response to G-CSF, cells were sampled at various time points after being transferred into the G-CSF-containing medium. Cells were stained with Wright–Giemsa solution, and morphological changes were evaluated using a microscope (Fig. 3A). With IL-3, the parental line GM-I62M and both transfectant lines GM-Lyn and GM-LynKN, showed immature myeloblastic morphologies. The morphology of GM-I62M cells cultured with G-CSF gradually changed and after about 12 days, a large portion of the cells showed the characteristic morphology of neutrophilic granulocytes with lobulated nuclei. In contrast, both the GM-Lyn and GM-LynKN cell lines showed neutrophilic morphology as early as 2 days after

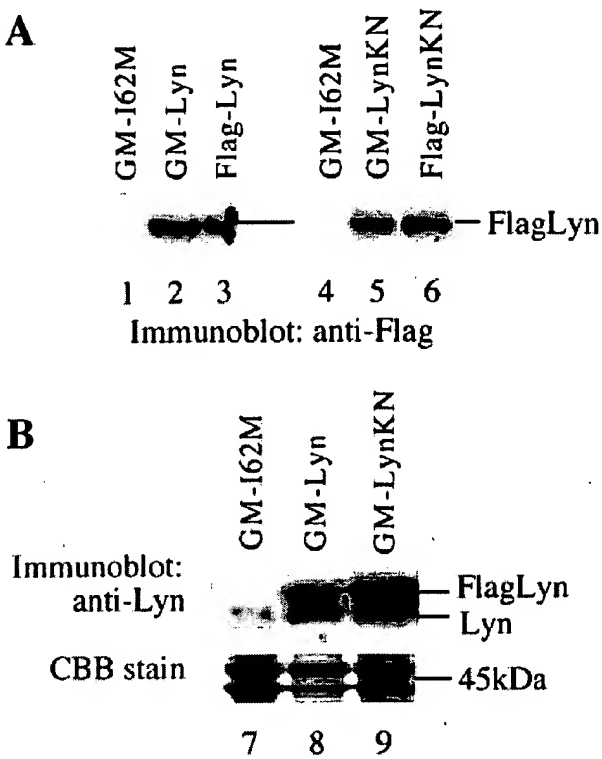

Fig. 1. Expression of Flag-Lyn and Flag-LynKN in stable transformants of GM-I62M. (A) Cell extracts (1×10^6 cell equivalents for GM-I62M and transformants, and 1×10^5 cell equivalents for COS-7 cells) were prepared from parental cells, GM-I62M (lanes 1 and 4), pBOS-FlagLyn transformant (GM-Lyn) (lane 2), and pBOS-Flag-LynKN transformant (GM-LynKN) (lane 5), which were cultured in RPMI 1640 with 10% fetal bovine serum and IL-3, and COS-7 cells transiently transfected with pBOS-FlagLyn (lane 3) and pBOS-Flag-LynKN (lane 6) as controls. Proteins were separated on SDS/10% polyacrylamide gels, followed by electroblotting on to GVHP membranes. Flag-tagged proteins on the membrane were decorated with anti-Flag M2 IgG and HRP-conjugated anti-mouse IgG and were visualized by enhanced chemiluminescence. (B) Cell extracts were prepared and their proteins separated on two sets of SDS/10% polyacrylamide gels as described above. Proteins on one gel were stained with Coomassie Brilliant Blue R250 (CBB), and stained bands of molecular mass ≈ 45 kDa are shown on the lower panel as loading controls. Proteins on another set of gels were analyzed by immunoblotting with anti-Lyn IgG and HRP-conjugated anti-rabbit IgG (upper panel).

being transferred to the G-CSF-containing medium, and most of the cells displayed a lobulated nucleus 5 days after being cultured with G-CSF. The quantitative data for the G-CSF-induced morphological changes are shown in Fig. 3B–E. Two other Lyn and LynKN transformants gave the same results (data not shown).

In a previous publication, a Lyn-deficient chicken B-lymphocyte cell line, DT40, expressing the human G-CSF receptor failed to respond to G-CSF with DNA synthesis as measured by a [^{3}H]thymidine-incorporation assay [17]. Therefore, we expected that GM-LynKN cells might show some defects in G-CSF-dependent [^{3}H]thymidine incorporation (Fig. 4), even though our long-term proliferation data showed no apparent defects (Fig. 2).

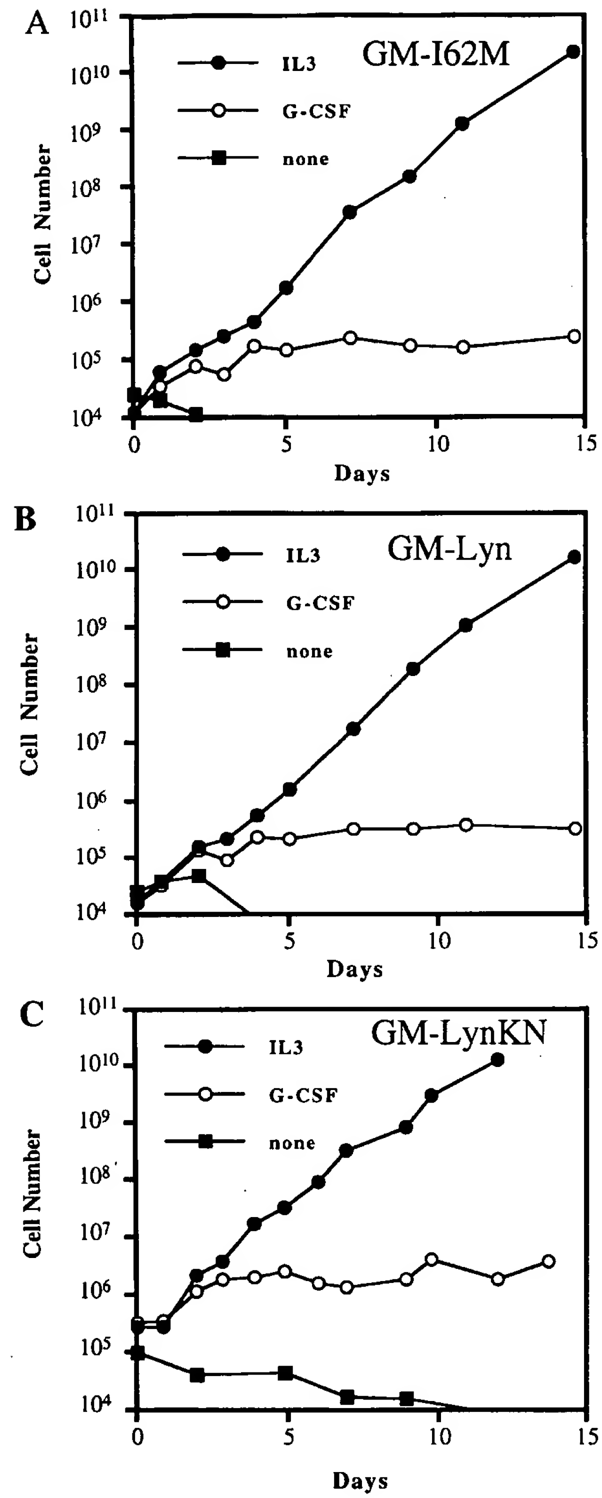

Fig. 2. G-CSF-dependent long-term growth of Lyn and LynKN transformants. The parental GM-I62M cells and GM-Lyn and GM-LynKN, maintained in medium containing 45 U·mL^{-1} IL-3, were washed twice, starved for 5 h in the factor-free medium and transferred to medium containing 45 U·mL^{-1} IL-3 (●), 150 U·mL^{-1} G-CSF (○), or no cytokine (■). Viable cells were counted by trypan blue staining under a microscope. (A) GM-I62M; (B) GM-Lyn; (C) GM-LynKN.

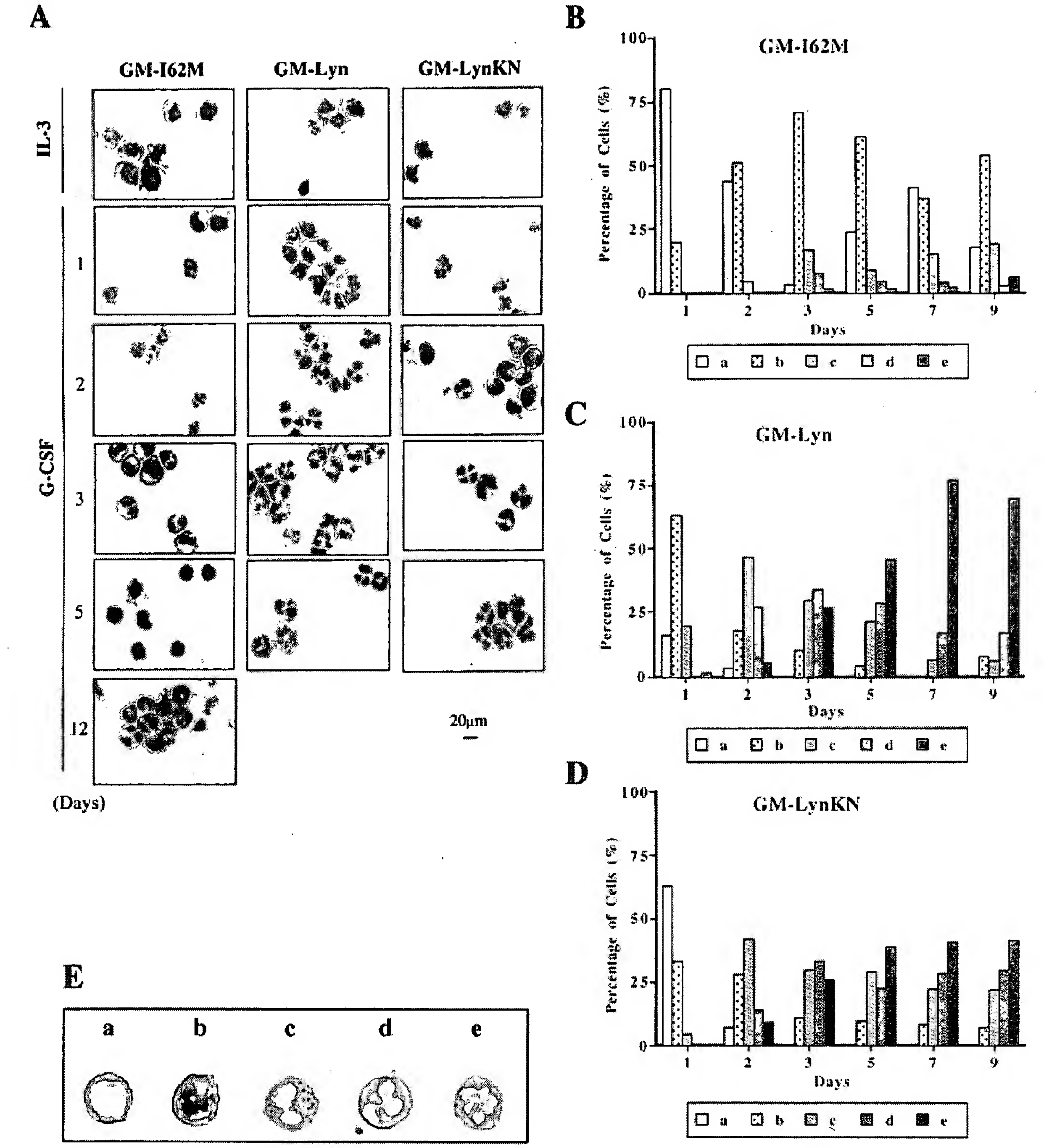

Fig. 3. G-CSF-induced morphological changes of GM-Lyn and GM-LynKN. (A) The parental GM-I62M cells and GM-Lyn and GM-LynKN were maintained in medium containing 45 U·mL^{-1} IL-3. After being washed with factor-free medium and starved for 5 h, the cells were cultured in the presence of G-CSF (150 U·mL^{-1}) for the indicated number of days. Cell morphology was visualized by Wright–Giemsa staining. Scale bar = 20 μm. (B–E) Quantitative analysis of the morphological changes. Fifty cells in each cell preparation in (A) were inspected under a microscope and classified into five categories (a-e) as shown in (E), depending on their degree of nuclear lobulation. (B) GM-I62M; (C) GM-Lyn; (D) GM-LynKN.

However, the G-CSF-induced thymidine incorporation of both the GM-Lyn and GM-LynKN cells appeared to be the same as the parental GM-I62M cells. Therefore, in contrast with the result in avian cells, overexpression of neither Lyn nor LynKN affected G-CSF-dependent DNA synthesis in the case of mouse myeloid cells.

MPO gene expression

Neutrophilic MPO is expressed when GM-I62M cells are cultured in the presence of G-CSF [26]. Expression of MPO is one of the markers of neutrophilic differentiation. Therefore, we examined the effects of Lyn and LynKN

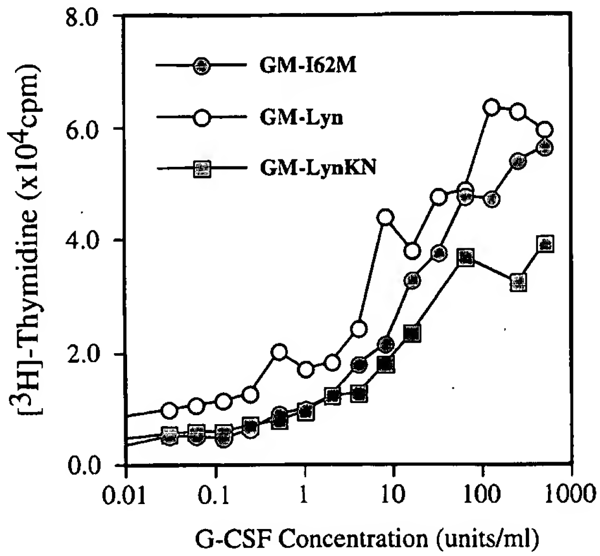

Fig. 4. G-CSF-dependent thymidine incorporation in the parental GM-I62M cells, GM-Lyn, and GM-LynKN. The cell lines were cultured in the various concentrations of G-CSF, and incorporation of [^{3}H]thymidine into the cells was measured. (◉) GM-I62M; (○) GM-Lyn; (▣) GM-LynKN.

overexpression on MPO gene expression. As shown in Fig. 5, when the parental GM-I62M cells were cultured in the presence of G-CSF, MPO mRNA was expressed after 2 days. Although its expression level in GM-Lyn cells was lower than in the GM-I62M cells, G-CSF-dependent expression of the MPO gene was evident in the GM-Lyn and GM-LynKN cells. As these Lyn-overexpressing cells started to show the nuclear morphological changes 48 h after being transferred to the G-CSF-containing medium

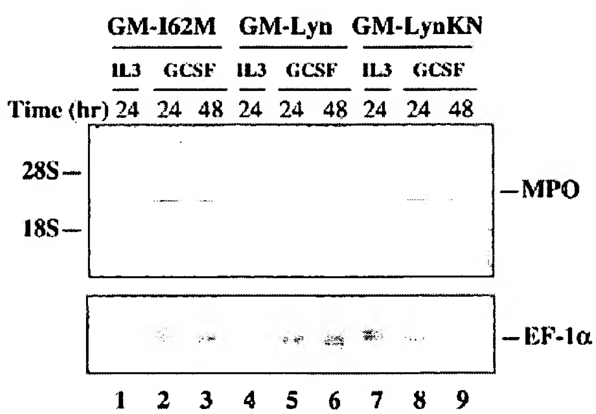

Fig. 5. Induction of MPO gene expression in GM-I62M, GM-Lyn, and GM-LynKN cells. Cells were maintained in medium containing 45 U·mL^{-1} IL-3. Cells were washed with factor-free medium and starved for 4 h, followed by incubation with either 45 U·mL^{-1} IL-3 for 24 h (lanes 1, 4 and 7) or 150 U·mL^{-1} G-CSF for 24 h (lanes 2, 5 and 8) or for 48 h (lanes 3, 6 and 9). Total RNA (10 μg·lane^{-1}) was analyzed by Northern-blot hybridization with ^{32}P-labeled murine MPO cDNA (upper panel). The same filter was stripped and hybridized with ^{32}P-labeled human EF-1α cDNA (lower panel). The positions of 28S and 18S ribosomal RNAs are indicated on the left.

(Fig. 3), G-CSF-dependent signaling pathways for nuclear lobulation and MPO gene expression appeared to be different, and the exogenous expression of Lyn or LynKN did not affect the G-CSF-dependent induction of MPO gene expression.

G-CSF-induced tyrosine phosphorylation of cellular proteins

Because overexpression of Lyn and LynKN accelerated G-CSF-induced nuclear lobulation, G-CSF-dependent signaling for nuclear lobulation was affected in these cells. Therefore, tyrosine phosphorylation of cellular proteins was examined by immunoblot analysis of total cell lysates prepared from GM-I62M, GM-Lyn, and GM-LynKN 2 min after stimulation with G-CSF. There was no apparent difference observed between the parental cells and the cell lines overexpressing Lyn or LynKN, except for the phosphorylation of Lyn itself (data not shown). Therefore, signaling molecules for nuclear lobulation are either unphosphorylated or phosphorylated but in undetectable amount in the cell lysates.

DISCUSSION

When neutrophil progenitor cells are stimulated with G-CSF, large numbers of proteins are tyrosine-phosphorylated, as observed by immunoblot analysis with an anti-phosphotyrosine IgG. These observations suggest that a number of protein tyrosine kinases are activated through the G-CSF-dependent signaling pathway. The roles played by the Jak family of kinases in cytokine signaling, including G-CSF signaling, have been extensively characterized. However, the functional roles of other protein tyrosine kinases in the G-CSF signaling pathway are not clear. An association between Lyn, a member of the Src kinase family, and the G-CSF receptor was reported, suggesting Lyn's involvement with G-CSF signal transduction. Moreover, a Lyn-deficient avian B-cell line has a defect in G-CSF-dependent proliferation, suggesting that Lyn is involved in mitogenic responses. To investigate the role of Lyn in the responses of mammalian granulocyte precursor cells to G-CSF, we expressed wild-type Lyn and its kinase-negative form, LynKN, at high levels in neutrophil progenitor cells, and examined the responses of these cells to G-CSF.

Unexpectedly, overexpression of both Lyn and LynKN in the neutrophil progenitor cells resulted in accelerated morphological changes with nuclear lobulation in response to G-CSF. These observations suggested that the Lyn protein but not its kinase activity is involved in G-CSF-dependent induction of nuclear lobulation. As Lyn is a Src tyrosine kinase, it has SH2 and SH3 domains besides its kinase domain. Therefore, overexpressed Lyn and LynKN appeared to work as adaptor proteins for G-CSF-dependent signal transduction in inducing nuclear lobulation. Alternatively, Lyn might have inhibited the signaling pathway that represses the induction of nuclear lobulation. In any case, its SH2 and/or SH3 domains appeared to be important for the protein–protein interactions needed to transduce the signals for G-CSF-dependent morphological changes. Immunoprecipitation of Flag-Lyn and Flag-Lyn-KN with an anti-Flag IgG yielded a few coimmunoprecipitating tyrosine-phosphorylated proteins. As yet, we have

not obtained evidence for direct interaction between Lyn and these phosphoproteins nor for their involvement in the signaling of nuclear lobulation. As the biochemical mechanisms underlying neutrophilic nuclear lobulation are still unclear, identification of proteins that interact with the Lyn SH2 and SH3 domains may provide great insight into these mechanisms.

In avian B cells reconstituted with the human G-CSF receptor, deficiency of Lyn as well as overexpression of a kinase-negative Lyn resulted in a defect in G-CSF-dependent thymidine incorporation [17]. However, in the murine granulocyte progenitor cell line GM-I62M, overexpression of a kinase-negative Lyn had only marginal effects on the G-CSF-dependent mitogenic responses. Therefore, in the murine cell line GM-I62M, either Lyn is not involved in G-CSF-dependent proliferation signaling or there are redundant mitogenic signaling pathways through the G-CSF receptor. We are currently examining the dispensability of Lyn in the G-CSF-dependent mitogenic response in other mammalian myeloid cells. Other possible mitogenic signaling pathways include activation of another Src kinase, Hck [15,43], STAT5 signaling [8,44,45], and Ras-MAPK/JNK/p38 pathways [21,32,46].

Fatty acylation of the N-terminus of Src family kinases is known to be essential for localization of the modified proteins to the plasma membrane and to plasma membrane rafts. Furthermore, S-acylation of the Src kinase, Lck, has been shown to be necessary for its localization to the plasma membrane and for signal transduction through the T-cell antigen receptor [47]. In our G-CSF signaling system, Flag-tag was fused to the N-terminus of wild-type and kinase-negative Lyn, which may have prevented fatty acylation of their own N-termini and also inhibited their targeting to plasma membrane. The negligible effects of the overexpression of kinase-negative Lyn on G-CSF-dependent mitogenic responses in our murine system could also be explained by the mislocalization of the tagged proteins without fatty acylation of their N-terminus. As overexpression of either Lyn or LynKN accelerated G-CSF-induced morphological changes during neutrophil differentiation, proteins that interacted with the overexpressed Lyn or LynKN appeared to be involved in the G-CSF-induced signaling for nuclear lobulation, wherever the overexpressed Lyn and LynKN were located. However, it will be important to determine the localization of Flag-tagged Lyn and its interacting proteins to clarify the signaling pathway for G-CSF-induced nuclear lobulation.

Overexpression of LynKN did not have much effect on other neutrophil differentiation markers tested, such as growth suppression and neutrophilic MPO gene expression. Therefore, G-CSF-dependent signaling for neutrophil differentiation consists of multiple pathways, one of which involves Lyn. Dominant-negative STAT3 has previously been shown to inhibit G-CSF-dependent growth suppression and nuclear lobulation, but to have no effect on MPO gene expression [48], suggesting that STAT3 is involved in the signaling pathway for growth arrest but not for the MPO gene expression and that nuclear lobulation might be a downstream phenotype of growth arrest. These observations agree with a report that 32D myeloid cells that overexpress Bcl2 without any cytokine stop dividing, survive, and undergo morphological changes to become neutrophilic granulocytes [49]. However, our data showing that overexpression of Lyn and LynKN in the G-CSF-responsive granulocyte precursor cells accelerated the neutrophilic morphological changes, which began before the growth arrest, suggest that the signaling pathways for growth suppression and induction of nuclear lobulation are independent and that Lyn is involved only in the latter.

Furthermore, as expression of the proto-oncogene, c-*myc*, correlated with the G-CSF-dependent growth properties in GM-I62M cells, c-*myc* expression was mediated by the activation of STAT3 [48], and STAT3 was activated, that is, phosphorylated on its tyrosine residue by G-CSF stimulation in all three cell lines (T. Yamamoto & H. Murakami, unpublished observation), G-CSF dependent induction of c-*myc* expression and its downregulation during growth suppression will take place in similar fashion in GM-I62M, GM-Lyn and GM-LynKN cells. However, as G-CSF-induced signaling for nuclear lobulation during neutrophil differentiation was stimulated by overexpression of Lyn and LynKN, the signaling pathway for nuclear lobulation was unlikely to be shared with that for the induction of c-*myc* expression or that for the G-CSF-dependent proliferation and growth arrest of these cells.

Our finding that overexpression of Lyn and LynKN accelerated the G-CSF-dependent morphological changes in neutrophil progenitors indicates that Lyn plays a role in G-CSF-induced signaling of neutrophil differentiation. Identification of proteins that interact with Lyn in a G-CSF-dependent manner will help to elucidate the molecular mechanisms of neutrophilic morphological changes, including nuclear lobulation.

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Cytokine receptor signalling

James N. Ihle

Many cell functions are regulated by members of the cytokine receptor superfamily. Signalling by these receptors depends upon their association with *Janus* kinases (JAKs), which couple ligand binding to tyrosine phosphorylation of signalling proteins recruited to the receptor complex. Among these are the signal transducers and activators of transcription (STATs), a family of transcription factors that contribute to the diversity of cytokine responses.

NUMEROUS aspects of lymphoid and myeloid cell function are controlled by a group of ligands termed cytokines, all of which signal through a related set of receptors. The conserved extracellular motifs of these receptors[1] are apparently derived from the fibronectin type III module. Different family members contain one to three chains, one or more of which generally have limited similarity in the membrane-proximal region (often referred to as box1/box2 motifs). All such receptors are associated with one or more members of a once obscure family termed the *Janus* kinases (JAKs). These kinases couple ligand binding to tyrosine phosphorylation both of various known signalling proteins and of a unique family of transcription factors termed the signal transducers and activators of transcription (STATs).

JAKs in cytokine signalling

The tyrosine kinases Fes, Lck, Hck, Lyn, Fyn and Syk are all activated or phosphorylated in response to individual cytokines[2,3], and the activation of Src family kinases by interleukin (IL)-2 is well documented[4]. Src kinases are not activated by most cytokines, however, and many IL-2 responses do not require Src. Similarly, the other kinases mentioned have not been widely implicated in cytokine signalling. By contrast, activation of all known cytokine receptors induces the tyrosine phosphorylation and activation of one or more JAK kinases associated with the receptor, and JAK activation is required for most, if not all, receptor functions.

The JAK family consists of Jak1, Jak2, Jak3 and Tyk2 (refs 2, 5), each of relative molecular mass in the range 120–130K. All have a carboxy-terminal kinase domain immediately preceded by a pseudokinase domain. No other recognizable protein motifs are present, although amino-terminal domains are conserved in the different family members. With the exception of Jak3, which is primarily expressed in haematopoietic cells, JAKs are ubiquitously expressed. The *Drosophila hopscotch* locus encodes a JAK homologue[6]; mutations at this locus cause developmental abnormalities, and a gain-of-function mutation encoded by the *Tum*^{l} allele causes a form of leukaemia in flies[7,8].

There are three patterns by which JAK kinases associate with receptors, depending on the receptor structure (Fig. 1). Several cytokines use single-chain receptors which associate with Jak2 (or, to a variable and lesser extent, with Jak1) by means of their conserved membrane-proximal domain. Association can be constitutive or enhanced by ligand binding. Receptor aggregation induces concomitant aggregation of Jak2, which is thought to allow transphosphorylation of the KEYY site in the kinase activation loop, dramatically increasing catalytic activity. Activated Jak2 could then phosphorylate itself, the receptor and cellular substrates recruited to the receptor complex. As this model predicts, mutations in the extracellular domain of the receptor leading to ligand-independent receptor aggregation also render Jak2 activation constitutive[9,10].

The IL-3 and IL-6 cytokine families typify the second association pattern. The receptors for IL-3, IL-5 and granulocyte/macrophage-colony-stimulating factor (GM-CSF) each consist of a ligand-binding α-chain associated with a common β_c-chain[11]. Jak2 associates with the membrane-proximal domain of the β_c-chain[12], aggregation of which mediates signal transduction. Similarly, the receptors for the cytokines IL-6, IL-11, CNTF, OSM and LIF all use ligand-specific binding chains that associate either with gp130 or the related signalling protein LIFR-β[13]. Unlike other receptor chains, gp130 associates with and activates Jak1, Jak2 and Tyk2 (refs 14, 15). Activation of Jak1 is essential for signalling and depends on the presence of either Jak2 or Tyk2 (ref. 16).

The IL-2 and interferon (IFN) receptor families typify the third association pattern, in which two chains are required for signalling. The IL-2 receptor consists of α-, β- and γ_c-chains[3], the cytoplasmic domains of the last two being required for signal transduction. The γ_c-chain also forms part of the receptors for interleukins 4, 7, 9 and 15, each of which also contains a ligand-specific α-chain related to the IL-2 receptor β-chain (or, in the case of IL-15, both the β- and γ_c-chains[17]). In each case, Jak1 associates with the membrane-proximal region of the IL-2 receptor β-chain or the ligand-specific α-chains, while Jak3 associates only with the shared IL-2 receptor γ_c-chain. Ligand-induced receptor aggregation then brings Jak1 and Jak3 together.

Similarly, the receptors for IFN-α/β and IFN-γ each contain at least two chains required for signal transduction. In the IFN-α/β receptor, Jak1 associates with β-chain[18] while Tyk2 associates with the α-chain[19]. In the IFN-γ receptor, R1 (the α-chain) associates with Jak1 (ref. 20) while R2 (the β-chain) associates with Jak2 (S. Pestka, manuscript submitted). Here, both JAKs are required for activation of either JAK[2]. The IL-10 receptor consists of a cloned chain related to the IFN receptors and a second, uncloned chain[21], which are thought to associate with Jak2 and Tyk2, the JAKs that are activated by IL-10.

In all cases, the primary function of the ligand is to mediate receptor aggregation and the concomitant homo- or heterotypic aggregation of JAKs. Curiously, only JAK2 is activated homotypically. Other JAKs may not autophosphorylate efficiently, as suggested by studies of Jak1 phosphorylation in IL-6 signalling, although any JAK autophosphorylates and becomes activated when overexpressed in insect cells (F. W. Quelle and J.N.I., unpublished observations). In some cases, the receptor structure may dictate the need for two JAKs, or both JAKs may be needed to form a high-affinity receptor complex[22].

Several observations underline the critical role of JAKs in signalling. Genetic selection of cells unable to respond to IFN produces cell mutants lacking Jak1, Jak2 or Tyk2, and addition of the missing JAK restores signalling[2,23]. Moreover, deletions or mutations affecting JAK association inactivate all receptor functions in all receptors examined. Deletions or mutations in the IL-2 receptor γ_c-chain that disrupt Jak3 association inactivate the receptor, producing X-linked severe combined immunodeficiency in humans[17], and kinase-negative Jak2 is a dominant suppressor of the cellular response to erythropoietin (Epo)[24] or IL-6[16]. Lastly, the absence of Jak3 in mice or humans is associated with deficiencies in lymphoid development which result in severe combined immunodeficiencies[87,90].

JAKs and cytokine-induced signalling

Like the receptor tyrosine kinases, cytokine receptors activate many signalling pathways, generally by means of phosphotyrosine residues, which are recognized by SH2 domains on the signalling molecules (Fig. 2). Most cytokines activate Ras by inducing phosphorylation of the adaptor protein SHC[25]. Other common targets include the p85 subunit of phosphatidylinositol

3-OH kinase (PI(3)K) and (less often) phospholipase C-γ1 (PLC-γ1), both of which (like SHC) are recruited to the receptor by virtue of their SH2 domain. Mutations disrupting JAK association with the receptor complex eliminate SHC and p85 phosphorylation[36,37], but whether JAKs directly phosphorylate SHC, PLC-γ1 or p85 is still unclear.

The receptor domains required to recruit various signalling proteins and the role of the proteins recruited can be assessed by the effects of mutations in the receptor. SHC and p85 phosphorylation, together with Ras activation, require the membrane-distal domain[26,28], which is not required for mitogenesis. In haematopoietic cells, Ras activation is thought to suppress apoptosis[29] and activate the transcription factor NF-IL-6 (ref. 30), it may also contribute to STAT activation. The function of PI(3)-K is unknown, but it may contribute to preventing apoptosis[31] and/or activate the Ras pathway by an SHC-independent mechanism[32,33].

Cytokines also induce tyrosine phosphorylation of Vav. Although Vav is not required for haematopoiesis[34], it is critical for signalling by the T- and B-cell antigen receptors in lymphoid cells[35-37]. With cytokine receptors, phosphorylation requires JAK activation[38] but only the membrane-proximal region of the receptor is needed, and Vav may be recruited to the receptor complex through phosphotyrosine residues on JAKs[39].

Among the cytokines, IL-4 is unusual in that it has no effect on Ras[40] and induces tyrosine phosphorylation of the protein insulin-receptor substrate-1 (IRS-1) or its relative IRS-2/4PS (ref. 41), which are thought to activate several signalling pathways[42]. These proteins associate with the IL-4 receptor α-chain, and mutations affecting this association eliminate a mitogenic response. Phosphorylation requires JAK activation[43], although it is unclear if IRS-1 or IRS-2 are JAK substrates. Growth hormone[44] and IL-9 (ref. 45) also induce phosphorylation of IRS-1 and IRS-2, respectively.

The role of Lck in signalling by the IL-2 receptor is still uncertain. Lck binds to the membrane-distal domain of the receptor's β-chain through its kinase domain[46], however, and deletions of this domain block IL-2 induced *c-fos* transcription. Syk, too, is constitutively associated with the membrane-proximal region of this chain[47]. The ancillary kinases may therefore phosphorylate the receptor so as to allow the recruitment of signalling proteins, or phosphorylate proteins recruited to the receptor complex that are not substrates for JAKs.

Cytokine-induced STAT activation

Cytokines also activate the STAT class of transcription facto STATs were first identified in studies of IFN-regulated ge expression[23], and Stats 1 and 2 are tyrosine-phosphorylated response to IFN-α/β. Phosphorylation triggers formation o complex with the DNA-binding protein p48, which moves the nucleus and activates transcription of genes bearing the int feron-response element (ISRE). Similarly, Stat1 phosphory tion induced by IFN-γ causes it to dimerize[48], move to nucleus and bind to the gamma-activated sequence (GAS) IFNγ-responsive genes. These findings suggested that other cy kines might also use STATs, a hypothesis which was rapi borne out by the cloning of the genes for Stats 3 (refs 49, 5 4 (refs 51, 52), 5 (ref. 53) and 6 (refs 43, 54). Indeed, virtua all cytokines appear to activate one or more STATs.

The STATs contain a carboxy-terminal SH2 domain, an SH like domain and several conserved amino-terminal regions, addition to a conserved region in the middle of the protein tl binds DNA[55]. Tyrosine phosphorylation of a carboxy-termi site mediates homo- or heterodimerization through the SI domains, triggering movement to the nucleus and DNA bindi Serine/threonine phosphorylation, possible mediated by MAP kinases[56,57], also plays a role in activation, linking ST/ and Ras activation.

STATs recognize related elements consisting of the inven repeat TTNCNNNAA. Binding and amplification reactic with random oligonucleotides show that the 'optimal' bindi site for Stats 1 and 3 is TTCC(C/G)GGAA[56], but preferi binding sites for each STAT can readily be identified amo known GAS sites by competition approaches[58]. The ability various STATs to heterodimerize may also influence th sequence specificity.

In all cases examined, mutations in cytokine receptors tl eliminate their association with JAKs abolish STAT phospho lation, and JAKs can perform the single tyrosine phosphory tion necessary to activate DNA binding by STATs *in vitro*. Individual JAKs exhibit no detectable preference for speci STATs *in vitro*, however, and their remarkable specificity *in v*. does not reflect kinase specificity.

Cytokine specificity and redundancy

Cytokines typically induce both overlapping and unique biolo cal responses, which reflect the different signalling pathw

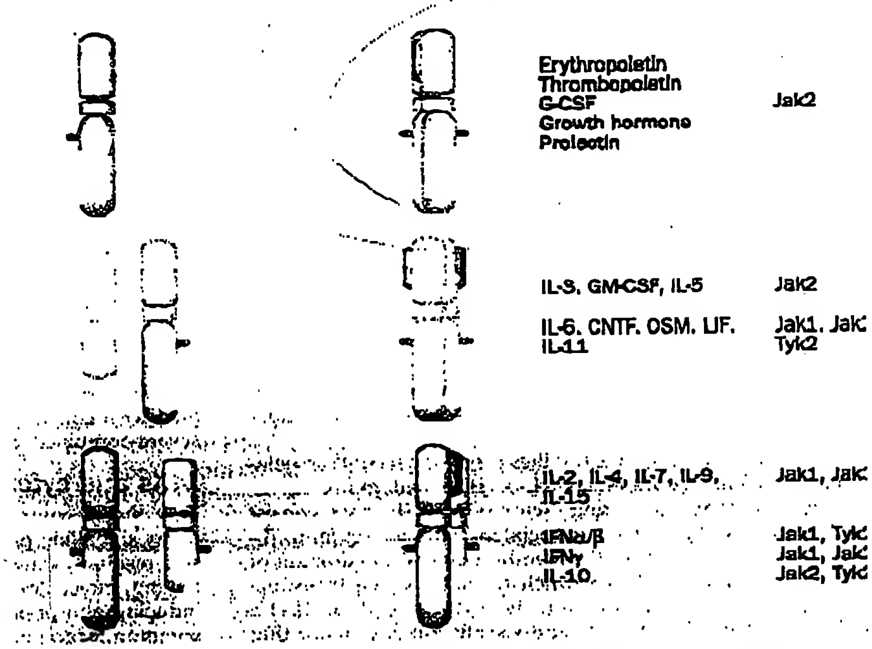

FIG. 1 Models for the association and activation of JAKs with and by different cytokine receptors. The first class of receptors (top) consists of single chains which primarily associate with Jak2 through a receptor membrane-proximal domain containing the box1/box2 motif (see also Fig. 2). The second class of receptors consists of two chains. One chain is required for ligand binding and a second shared chain is required for signalling. The shared signalling chain associates with one or more of the JAKs, as indicated, through a membrane-proximal domain containing the box1/box2 motif. The third class of receptors contain at least two chains, two of which are required for signalling. In these cases, JAKs associate with both signalling chains. In the IL-2 receptor subfamily, the shared γ-chain associates with Jak3 while the IL-2 receptor β-chain, or corresponding α-chains, associates with Jak1 through the membrane-proximal domain containing the box1/box2 motif. Among the IFN receptors, the individual chains associate with the JAKs indicated. In all cases, ligand-induced receptor aggregation is proposed to aggregate the associated Jaks and allow their activation by transphosphorylation. The JAKs are indicated in yellow.

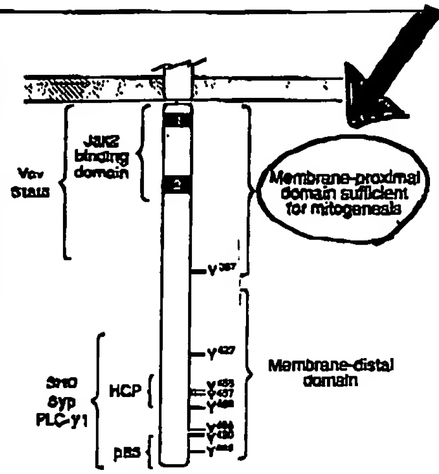

FIG. 2 Signalling proteins associating with the erythropoietin receptor. The Epo receptor associates with and activates Jak2. Jak2 association occurs through the membrane-proximal region in the box1/box2-containing domain. In response to Epo, tyrosine phosphorylation of the receptor occurs at sites in the membrane-distal region, together with phosphorylation of Vav, Stat5, SHC, PLC-γ1, Syp and p85. The membrane-proximal region of the receptor is necessary and sufficient for Vav and Stat5 phosphorylation as well as for a mitogenic response. The membrane-distal region of the receptor, which is dispensable for mitogenesis, is required for the recruitment and subsequent phosphorylation of SHC, Syp, PLC-γ1 and p85. Tyrosine phosphorylation of the distal region of the receptor also creates binding sites for HCP and recruits the phosphatase to the receptor complex in which it negatively influences receptor activity.

involved. In T cells, for example, IL-2, 4 and 9, all of which utilize the IL-2 receptor γ-chain, activate Jak1 and Jak3, whereas IL-10 and 12 activate Jak1 and Tyk2 and Jak2 and Tyk2, respectively[91,92]. The STATs show an even greater range of responses, each cytokine activating a unique set. Different cytokines may also activate Ras, IRS proteins or other cytoplasmic kinases. Some of the differences in the effects of these cytokines[60] will certainly reflect the constellation of signalling pathways they activate, and mutants that uncouple activation of the individual pathways will be needed to dissect them.

Several cytokines have remarkably similar biological activities, partly because their receptors contain common signalling chains. Some of the similarities, however, reflect the STATs involved. G-CSF, for example, shares many properties with IL-6 when examined on the same cells, largely because both activate Stat3.

The specificity with which STATs and other proteins are activated relies on the specificity with which they are recruited to the receptor complex. Often this depends upon recognition of phosphotyrosine docking sites on the receptor by the STAT SH2 domain[43,61]. Docking sites exist on the β-chain of the IL-2 receptor and the α-chains of the IL-4 and IL-10 receptors for Stats 5, 6 and 3 respectively (refs 43, 62) and R. D. Schreiber, submitted). Indeed, addition of relatively short peptides containing a docking site to a receptor allows Stat recruitment and activation[61], and switching STAT SH2 domains produces a corresponding change in the receptors required to activate them[63].

Other receptors, such as those for growth hormones[64] can recruit and activate STATs without being phosphorylated, either by means of an alternative type of association, or by means of an undiscovered receptor chain. The potential complexity of STAT recruitment is illustrated[65] by Stat2's role in recruiting and phosphorylating Stat1 in response to IFN-α/β. In this case, Stat2 is thought to provide docking sites for Stat1 after first binding to the receptor complex and being phosphorylated itself, indicating that Stat1/Stat2 heterodimerization may be important.

Biological functions of the STATs

STATs regulate the expression of a wide range of genes. Transcription of many genes controlled by IFN[23], some of which are involved in antiviral responses, requires Stat2 and/or Stat1. Similarly, IL-6-induced expression of the acute-phase response genes requires Stat3 (ref. 13), whereas Stat5 mediates prolactin-induced transcription of several proteins secreted in milk[53]. Stat6 binds to an element in the immunoglobulin locus required for IL-4-induced class switching[66,67], and mediates IL-4-induced upregulation of the major histocompatibility complex (MHC) class II antigen, various immunoglobin receptors and other cell surface proteins.

Although the STATs are ubiquitously expressed (with the exception of Stat4: ref. 51), they probably regulate different genes in different cell types. Stat5 induces transcription of the β-casein and whey acidic protein (WAP) genes in mammary gland cells, for example, but not in myeloid cells. Like many transcription factors, STATs probably act as components of multi-protein transcriptional complexes, which may also contain cell lineage- or differentiation-specific components. Stat5-induced transcription of the WAP gene also requires a binding site for nuclear factor 1 (ref. 68).

STATs appear only to be involved in functional (as opposed to mitogenic) responses to cytokines, as IFNs do not generally induce proliferation and, indeed, are typically antiproliferative[69]. Moreover, IL-6 (ref. 61), IL-2 (ref. 62) and IL-4 (ref. 43) mutant receptors can trigger cell proliferation without activating Stats 3, 5 and 6 respectively.

Phosphatase regulation of cytokine signalling

Cytokine receptors are also negatively regulated, in part by haematopoietic cell phosphatase (HCP, also termed PTP-1C or SH2-PTP1). HCP contains a carboxy-terminal catalytic domain specific for tyrosine and two amino-terminal SH2 domains, the first of which facilitates recruitment of HCP to activated receptors, including *c-kit*[70], the IL-3 receptor β-chain[71], the B-cell antigen receptor[72] and the Epo receptor[73,74]. The mouse mutant *motheaten*, which lacks functional HCP, suffers from numerous haematopoietic abnormalities attributable to cell overproliferation[75,76], and over- or underexpression of HCP suppresses or enhances cytokine responses[71]. In some cell lines, recruitment of HCP to the receptor complex is associated with dephosphorylation of Jak2 (ref. 74), suggesting that JAKs are critical substrates.

By contrast, the related phosphatase Syp (also termed SH-PTP2 or PTP-1D) is thought to stimulate signalling. Syp too contains two SH2 domains which allow it to bind to the receptors for IL-6 and other cytokines[61]. It is then tyrosine-phosphorylated, although it is unclear whether a JAK is responsible. The result is to activate MAP kinase, perhaps by binding Grb2 and activating the Ras pathway[77]. It may also activate Src-family kinases by dephosphorylating their carboxy-terminal phosphotyrosines.

As already noted, the specificity of signalling generally depends on the receptor complex and not the JAKs. Indeed, overexpression of JAKs promiscuously activates endogenous JAKs and STATs. To retain specificity, activated JAKs that dissociate from the complex must thus be inactivated, perhaps by cytoplasmic phosphatases such as HCP.

Other receptor systems

Although they are best known for their role in cytokine-receptor signalling, JAKs and STATs also participate in signalling by other receptors. EGF-induced *c-fos* expression requires STATs.

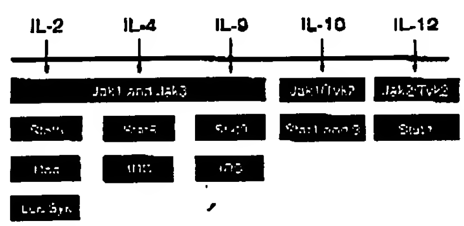

FIG. 3 Diversity in cytokine-induced T-cell signalling. The spectrum of JAKs and signalling proteins activated by the individual cytokines is indicated. No information is available regarding the activation of the Ras pathway or IRS-2 phosphorylation by IL-10 or IL-12. Similarly, IL-4, IL-9, IL-10 and IL-12 have not been reported to activate other cytoplasmic kinases.

[redacted]

which are thought to be recruited to a *cis*-inducible element in the promoter[49,78,80] following. SH2-mediated interactions with the phosphorylated EGF receptor, and subsequent tyrosine phosphorylation. The purified EGF receptor can indeed directly phosphorylate Y701 of Stat1 (ref. 59), and it will be important to determine which other tyrosine kinases share this capacity.

JAKs and STATs are also activated by angiotensin II, whose receptor is a member of the G-protein-coupled receptor family[81,82]. Several such receptors trigger tyrosine phosphorylation of focal adhesion kinase[83] or Src kinases[84] on binding ligand, and it will be interesting to determine whether all members of this family activate JAKs and STATs.

Cellular transformation

JAKs and/or STATs may also contribute to cellular transformation. As already mentioned JAK mutations in *Drosophila* can cause transformation[7,8], and mutations leading to constitutive dimerization of cytokine receptors (and thus JAK activation) may cause transformation. Moreover, HTLV-1 infection is thought to lead, whether directly or indirectly, to a similar receptor-mediated activation of the pathway[85]. So far, however, JAKs have not been shown to transform mammalian cells.

The observation that Stat3 is constitutively phosphorylated in v-*src*-transformed cells has also been taken to support a role for STATs in transformation[86]. v-Src phosphorylates a variety of cellular substrates, however, only some of which are involved in transformation, and as noted above, there is no other evidence that Stat3 is involved in mitogenic responses.

Future directions

Although JAKs play a critical role in cytokine-induced signalling, relatively little is known about their structural domains. JAKs are phosphorylated at several unidentified sites in response to cytokines in addition to the activation loop, which are probably important in recruiting additional signalling proteins to the receptor complex. Moreover, the domains that associate with the membrane-proximal regions of the receptors have yet to be identified, and the functions of the JAK-homology domains and the pseudokinase domain are unknown.

Other properties of the STATs also remain unclear. Of particular interest is the mechanism by which STAT dimers translocate to the nucleus; is it passive or facilitated? In the IFNα/β-induced transcription complex, p48 is essential. This protein is a member of a family that includes IRF-1 and ICSBP: do these proteins functionally associate with other members of the STAT family? Other questions, such as the biological role of each JAK and STAT, will be resolved as mice lacking functional genes for the different JAKs and STATs (or combinations thereof) become available.

Finally, most cytokines induce cell-cycle progression, which is generally assumed to require transcriptional activation of c-*myc*. The involvement of several signalling pathways in these responses, including the Ras pathway, has been ruled out by the use of receptor mutants, and the identity of the pathways involved will be of great interest. The central role of JAKs and STATs in the cellular responses controlling numerous aspects of lymphoid and myeloid function also makes them attractive targets for drug development. □

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Immunology

Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family

(signal transduction)

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ABSTRACT **Interleukin 6 (IL-6) signal is transduced through gp130 that associates with a complex of IL-6 and IL-6 receptor. Truncations or amino acid substitutions were introduced in the cytoplasmic region of human gp130, and the mutant cDNAs were transfected into murine interleukin 3-dependent cells to determine amino acid residues critical for generating the IL-6-mediated growth signal. In the 277-amino acid cytoplasmic region of gp130, a 61-amino acid region proximal to the transmembrane domain was sufficient for generating the growth signal. In this region, two short segments were significantly homologous with other cytokine-receptor family members. One segment is conserved in almost all members of the family, and the other is found especially in granulocyte colony-stimulating factor receptor, interleukin 2 receptor β chain, erythropoietin receptor, KH97 (a granulocyte/macrophage colony-stimulating factor receptor-associated molecule), and interleukin 3 receptor. gp130 molecules with mutations in either of these two segments could not transduce growth signal. Loss of signal-transducing ability of gp130 with such a mutation coincided with disappearance of IL-6-induced tyrosine phosphorylation of gp130.**

Interleukin 6 (IL-6) acts on a wide variety of cells and exerts multiple functions, such as growth promotion, growth inhibition, differentiation, and cell-specific gene expression (1, 2). To elucidate the mechanism of this functional pleiotropy of IL-6, we have shown that the IL-6 receptor (IL-6-R) system comprises two cell-surface molecules: an IL-6-binding protein (IL-6-R) and a signal transducer, gp130. Upon binding of IL-6, IL-6-R becomes associated extracellularly with gp130 to form high-affinity IL-6-binding sites, and gp130 transduces the signal (3, 4). Soluble IL-6-R (sIL-6-R) lacking transmembrane and cytoplasmic regions can associate with gp130 in the presence of IL-6 and mediate the IL-6 signal (3, 5).

Most receptors for cytokines involved in growth and differentiation of hematopoietic lineage cells or their associate molecules, like gp130, are structurally similar and belong to the cytokine receptor family (4, 6–8). The homologous segment in the extracellular region comprises two fibronectin type III modules and includes four conserved cysteine residues in the amino-terminal module and a Trp-Ser-Xaa-Trp-Ser motif in the other one. On this basis, it has been suggested that members of the cytokine receptor family have evolutionarily emerged from a common ancestral molecule (8, 9). For the cytoplasmic region no strict consensus sequences were seen, and no tyrosine kinase domain existed. However, the cytoplasmic regions of IL-2-R β chain, IL-3-R, IL-4-R, IL-7-R, granulocyte colony-stimulating factor receptor, erythropoietin receptor (Epo-R), and gp130 possessed a segment rich in serine residues (4, 10–16). And some sequence similarities were reported among IL-2-R β chain, Epo-R, IL-3-R (10, 12), granulocyte colony-stimulating receptor, IL-3-R, IL-4-R, and Epo-R (15). Functionally important regions in the cytoplasmic region of some cytokine-receptor family members were reported. For example, in the IL-2-R β chain, a hydrophobic amino acid in the serine-rich region was important for IL-2 signal transduction (17, 18). In the Epo-R, there was a negative regulatory domain in the carboxyl-terminal end of the cytoplasmic region, and a ≈100-amino acid segment proximal to the transmembrane domain was critical for the erythropoietin-dependent growth (19). For gp130, in addition to the serine-rich region, the consensus sequence for nucleotide binding commonly found in protein kinases and GTP-binding motif-like sequences were observed in the cytoplasmic region (4).

In our study to help understand IL-6 signal transduction through gp130, truncations or amino acid substitutions were introduced in the cytoplasmic region of gp130. First we describe the presence of a homologous region, located proximal to the transmembrane domain, which includes two segments highly conserved in the cytokine-receptor family. Using gp130 mutants, we show that, not a serine-rich region or GTP-binding motif-like sequences but instead, this homologous region is critical for generating the IL-6-mediated growth signal. This work raises the possibility that a similar mechanism might be involved in signal transduction through members of the cytokine-receptor family. We further discuss IL-6-induced tyrosine phosphorylation of gp130.

MATERIALS AND METHODS

Plasmid Construction and Transfection. pZip130 has been reported (4). For construction of plasmids pZipIC141 and pZipIC93, pUC18, including the entire human gp130 cDNA, was linearized at the cytoplasmic region with *Acc* I or *Eco*T22I, respectively, and a universal terminator (Pharmacia) was inserted. Each mutated gp130 cDNA fragment was inserted into the pZipNeoSV(X) expression vector. For construction of the other plasmids, site-directed mutagenesis was done with an oligonucleotide-directed *in vitro* mutagenesis system (Amersham), according to the manufacturer's directions. Briefly, M130, containing the entire gp130-coding sequence in M13mp18, and M130IC65, constructed from M130 to possess the termination codon at the 66th position in the cytoplasmic region of gp130, were first prepared. pZipIC10, pZipIC38, pZipIC54, pZipIC61, pZipIC65, and pZ-

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Abbreviations: IL-2, -3, -4, -5, -6, -7, interleukins 2–7, respectively; IL-6-R, interleukin 6 receptor (attachment of an R to any interleukin abbreviation refers to its receptor); sIL-6-R, soluble IL-6-R; Epo-R, erythropoietin receptor.

ipPP were constructed from M130; pZipKK, pZipIW, pZipPOS, and pZipNEG were from M130IC65. An *Acc* II fragment containing a gp130-coding region with a specific mutation was inserted into pZipNeoSV(X). Transfectants were prepared and examined for the expression of gp130 by flow cytometric analysis (3, 4).

Internal Labeling and Immunoprecipitation. The internal labeling and immunoprecipitation were done as described (3). Briefly, [^{35}S]methionine-labeled cells (1×10^7) were stimulated with human sIL-6-R (5 μg/ml) plus IL-6 (500 ng/ml) at 37°C for 10 min. Digitonin lysates were immunoprecipitated with anti-human IL-6-R antibody MT18 (20).

Cell Proliferation Assay. Cells (1×10^4 in 0.1 ml) were cultured in triplicates with human IL-6 in the presence of sIL-6-R (2.5 μg/ml) in RPMI 1640 medium/10% fetal calf serum in 96-well microplates for 40 hr. Cells were pulse-labeled with [^{3}H]thymidine (1 nCi per well; 1 Ci = 37 GBq) for 8 hr, and the incorporated radioactivities were measured. Values obtained with sIL-6-R alone were 138 ± 21 cpm–367 ± 33 cpm, depending on transfectants used.

Immunoblot Analysis of gp130. Cells (1×10^8) were preincubated in serum-free Eagle's minimal essential medium for 4 hr and stimulated with sIL-6-R (10 μg/ml) plus IL-6 (2.5 μg/ml) for 10 min. Nonidet P-40 lysates (3) were immunoprecipitated with anti-gp130 antibody AM66 and subjected to immunoblot analysis by using polyclonal anti-phosphotyrosine antibody (21).

RESULTS

Preparation of Transfectants with gp130 Mutants. From the deduced amino acid sequence of gp130 (4), we observed the following features in the cytoplasmic region (see Fig. 1*A*): (*i*) The middle of the cytoplasmic region contains a serine-rich region, as also seen in several other cytokine receptors (IL-2-R β chain, IL-4-R, and granulocyte colony-stimulating factor receptor. (*ii*) The amino acid sequence (in single-letter code) GPGTEGQV fits the consensus sequence for nucleotide binding, GXGXXGXV, commonly found in protein kinases (27). (*iii*) Four stretches of amino acid sequences, GPGTEGQ, DAFG, NKRD, and EVSA seem partially to fit the consensus elements reported as required for GTP binding in *ras* and *ras*-related proteins [GXXXXGK, DXXG, NKXD, and EXSA (28, 29)]. (*iv*) The ≈60-amino acid segment located proximal to the transmembrane domain possessed a sequence similarity with several other members of the cytokine-receptor family (as aligned in Fig. 1*B*). In this homologous segment, two stretches of amino acids are highly conserved. One comprises a Pro-Xaa-Pro sequence and a preceding cluster of hydrophobic amino acids (box 1 in Fig. 1*B*). The Pro-Xaa-Pro sequence exists in all receptors listed in Fig. 1, except that IL-7-R possesses Pro-Xaa-His, instead. The other homologous segment (box 2) begins with a cluster of hydrophobic amino acids and ends with one or two positively charged amino acids. In the middle of box 2, some

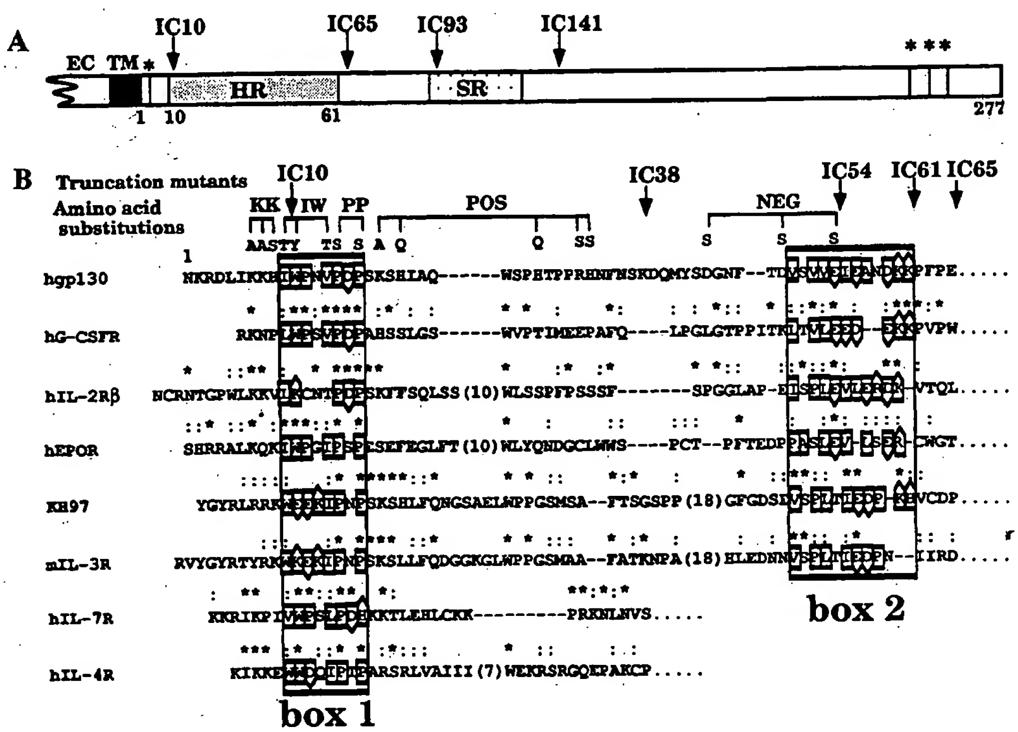

FIG. 1. Schematic structure and sequence alignment of the cytoplasmic region of human gp130. (*A*) Schematic depiction of the cytoplasmic region of human gp130. EC, extracellular region; TM, transmembrane domain; SR, serine-rich region; HR, homologous region (see Fig. 1*B*). *, GTP-binding motif-like sequences (from left, NKRD, EVSA, DAFG, and GPGTEGQV; the latter also fits the consensus for nucleotide binding). Arrows with the mutant names indicate where termination codons were introduced. (*B*) Sequence alignment of cytoplasmic region of gp130 with other cytokine-receptor family members. A part of the cytoplasmic region, located near the transmembrane domain, of the following molecules are aligned: human gp130 (4), human granulocyte colony-stimulating factor receptor (hG-CSFR) (22), human IL-2 receptor β chain (11), human erythropoietin receptor (hEPOR) (23), KH97 (24), mouse IL-3 receptor (12), human IL-7 receptor (14), and human IL-4 receptor (25). The receptors such as IL-5-R, IL-6-R, and granulocyte/macrophage colony-stimulating factor receptor having a relatively short cytoplasmic region that might not transduce the signal are omitted. Two highly conserved segments are boxed (boxes 1 and 2). In these boxes, amino acids are classified into four groups, according to the chemical characteristics of their side chains (26): nonpolar (boxed)—A, I, L, M, F, P, W, and V; polar but uncharged (unboxed)—N, C, Q, G, S, T, and Y; negatively charged (▯)—D, and E; and positively charged (▯)—R, H, and K. Identical amino acids with gp130 are marked as (*), and similar amino acids according to above classification are marked as (:). Gaps are introduced to maximize homology (– and numbers in parentheses). Positions of truncations and amino acid substitutions are indicated above gp130 sequence with the mutant names. The first amino acid in the cytoplasmic region is numbered 1.

negatively charged amino acids are clustered or harbored in mostly hydrophobic amino acids. The box 2 is conserved especially in gp130, granulocyte colony-stimulating factor, IL-2-R β chain, Epo-R, KH97, and IL-3-R (the last positively charged amino acid in box 2 is missing in IL-3-R). This similarity is conserved over the species (human and mouse) in each of the above receptors (data not shown). Besides the two boxes, tryptophan (W) and phenylalanine (F) residues are conserved (at positions 25 and 35, respectively, (see Fig. 1*B*).

On the basis of these structural features, we first prepared gp130 mutants carrying truncations of various numbers of amino acids from the carboxyl-terminal end. Translational termination codons were introduced at the positions indicated by arrows in Fig. 1*A*. gp130IC141 lacked three of the four GTP-binding motif-like sequences, and gp130IC93 was devoid of the serine-rich region. gp130IC65 contained a short cytoplasmic region but long enough to include the homologous region described above with four additional amino acids. gp130IC10 lacked almost all the cytoplasmic amino acids, except 10 [numbers following IC (intracytoplasmic) correspond to the number of remaining amino acids in the cytoplasmic region]. BAFB03 cells were transfected with these gp130 mutant cDNAs or the expression vector pZipneoSV(X) alone. The resulting transfectants—BAFIC141, BAFIC93, BAFIC65, BAFIC10, and BAFneoR—were obtained.

Association of Mutant gp130 Molecules with sIL-6-R and Their Function. We examined whether truncated gp130 molecules could associate with IL-6-R in the presence of IL-6. The above-mentioned five transfectants and BAF130 cells transfected with wild-type human gp130 cDNA (4) were metabolically labeled and incubated with a mixture of IL-6 and sIL-6-R because BAFB03 and its transfectants expressed no IL-6-R. Fig. 2 shows that the truncated gp130 molecules, as well as the wild-type gp130, were coprecipitated with sIL-6-R. The results indicated that gp130 mutants could normally associate with the complex of IL-6 and sIL-6-R, even when only the 10 amino acids remained in the cytoplasmic region.

We then examined whether these mutant gp130s could transduce the growth signal. Cells were incubated with sIL-6-R and IL-6, and the incorporation of the [^{3}H]thymidine was measured. Fig. 3 shows that BAFIC141, BAFIC93, BAFIC65, and BAF130 could dose-dependently respond to IL-6 in the presence of sIL-6-R, whereas BAFIC10 and BAFneoR could not. These results indicated that neither the serine-rich region nor three of the four GTP-binding motif-like sequences in gp130 were required for transduction and that the cytoplasmic homologous region of gp130 was enough for generating the IL-6-mediated growth signal in BAFB03 cells.

Function of gp130 Molecules Carrying Mutations in the Homologous Region. We further examined which part of the homologous region was critical for generating the growth signal in BAFB03 cells. Within the two conserved boxes, the Pro-Xaa-Pro sequence in box 1 is present in all receptors shown in Fig. 1*B*, except IL-7-R. The two prolines in this Pro-Xaa-Pro sequence were substituted by serines. This gp130PP mutant possessed the complete cytoplasmic region except for these two substitutions. All other amino acid substitutions were introduced in gp130IC65, which possessed 65 amino acids in the cytoplasm. In gp130IW mutant, three conserved hydrophobic amino acids, isoleucine, tryptophan, and valine, in box 1 that preceded the Pro-Xaa-Pro sequence, were substituted by threonine, tyrosine, and threonine, respectively. In gp130KK mutant, three successive positively charged amino acids, lysine, lysine, and histidine, just amino-terminal outside box 1, were substituted for by alanine, alanine, and serine, respectively. For box 2 translational termination codons were introduced at the carboxyl-terminal edge or in the middle of this box (gp130IC61 and gp130IC54, respectively). A mutant cDNA with a termination codon between boxes 1 and 2 was also prepared (gp130IC38). By using BAFB03 transfectants with the above cDNAs, these mutant gp130 molecules were revealed to retain their ability to associate with a complex of IL-6 and sIL-6-R (data not shown). From the cell-proliferation assay shown in Fig. 4, BAFPP cells with mutant gp130 cDNA, in which the highly conserved Pro-Xaa-Pro sequence was changed to Ser-Xaa-Ser but the rest of the 277-amino acid cytoplasmic region was

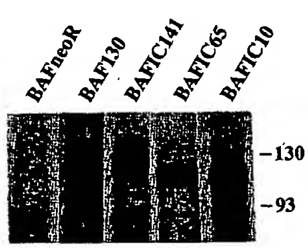

FIG. 2. Mutant molecules can associate with sIL-6-R in the presence of IL-6. BAFB03 transfectants were metabolically labeled and stimulated with sIL-6-R plus IL-6. Cells were lysed with digitonin and immunoprecipitated with the anti-IL-6-R monoclonal antibody MT18.

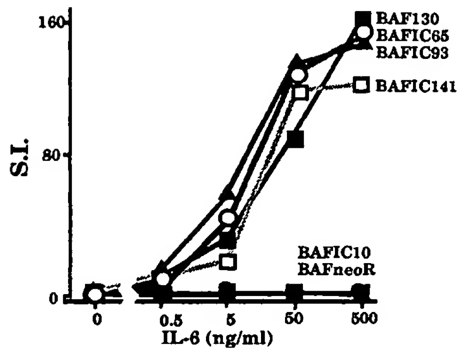

FIG. 3. Neither the serine-rich region nor three of the four GTP-binding motif-like sequences in gp130 were required for IL-6-mediated growth. BAFB03 transfectants were cultured with various concentrations of IL-6 in the presence of sIL-6-R. Incorporated [^{3}H]thymidine was measured. Data represent the stimulation index (S.I.), calculated from the incorporated radioactivity without IL-6.

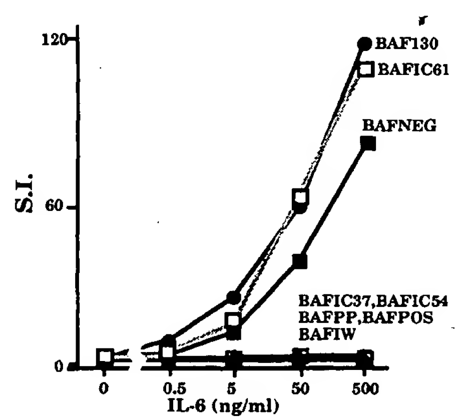

FIG. 4. Functional assay of gp130 molecules carrying mutations in the homologous region. BAFB03 transfectants expressing gp130 molecules with mutations in the homologous region were examined for the IL-6-dependent growth, as in Fig. 3. S.I., stimulation index.

intact, could not respond to IL-6 in the presence of sIL-6-R. Disturbance of the conserved three hydrophobic amino acids in box 1 also completely abolished the gp130-mediated proliferative response (see BAFIW cells in Fig. 4). In contrast, when three successive positively charged amino acids just outside box 1 were exchanged for uncharged ones, gp130 molecule carrying this mutation could normally transduce the growth signal (BAFKK cells, data not shown).

These results indicated that the box 1 segment in gp130, which is conserved in all members of the cytokine-receptor family, has a critical role in IL-6 signal transduction. The functional significance of the distal conserved segment (box 2) was also shown because BAFIC61 could respond to IL-6 in the presence of sIL-6-R, but BAFIC54 and BAFIC37 could not. These results indicated that the seven successive amino acids, IEANDKK, in box 2 were critical for generating the IL-6-mediated growth signal and that the 61-amino acid cytoplasmic portion was enough for signal transduction.

A 32-amino acid region between boxes 1 and 2 contained 9 charged amino acids. All 6 positively charged amino acids were located in the amino-terminal half of this region, and all 3 negatively charged amino acids were in the rest of the region. We prepared two mutants according to this feature. In one mutant, gp130POS, all the above-mentioned positively charged amino acids except the last lysine were substituted for by alanine, glutamine, or serine, so that the charges were lost but predicted α-helical and β-sheeted structures would not be greatly changed. In the other mutant, gp130NEG, the last 2 of the 3 negatively charged amino acids together with 1 glutamic acid residue (also a negatively charged amino acid) in box 2 were subjected to a similar substitution (see Fig. 1*B*). Fig. 4 shows that, in contrast to the fact that BAFPOS cells expressing the former mutant gp130 did not show any IL-6-dependent proliferative response, BAFNEG cells did show this response.

Phosphorylation of gp130. Some cytokines, such as IL-2, IL-3, and IL-7, phosphorylate tyrosine in cellular proteins, including the receptor (30–32). We examined whether gp130 was tyrosine-phosphorylated in response to IL-6. BAF130 cells were stimulated with a complex of IL-6 and sIL-6-R. Immunoprecipitated gp130 was immunoanalyzed by using polyclonal anti-phosphotyrosine antibody. Fig. 5 shows that gp130 molecule was tyrosine-phosphorylated after BAF130 cells were stimulated with a complex of IL-6 and sIL-6-R. When we used BAFPP cells expressing mutant gp130 in which two prolines of the highly conserved Pro-Xaa-Pro sequence were exchanged for serines, this mutant gp130 molecule was not tyrosine-phosphorylated after IL-6-stimulation. This mutant gp130 molecule possessed the entire cytoplasmic region, except two amino acid substitutions and showed no IL-6-induced tyrosine-phosphorylation in accordance with the loss of IL-6 responsiveness.

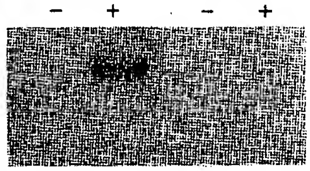

FIG. 5. gp130 was tyrosine-phosphorylated by IL-6 stimulation. BAF130 and BAFPP cells were stimulated (+) or unstimulated (−) with IL-6 plus sIL-6-R. Immunoprecipitated gp130 molecules were examined for tyrosine phosphorylation by immunoblot analysis. A comparable amount of gp130 was transferred in each lane as detected by anti-gp130 monoclonal antibody AM82 (ref. 4) (data not shown).

DISCUSSION

In this study, we showed that 61 of 277 amino acid residues in the cytoplasmic region of gp130 were sufficient to generate a growth signal in BAFB03-derived transfectants. This 61-amino acid segment did not include (*i*) the serine-rich region, (*ii*) the nucleotide-binding consensus GXGXXGXV, or (*iii*) three of the four GTP-binding motif-like sequences. The dispensability of the serine-rich region of gp130 is in contrast to the observation with IL-2-R β chain; this latter molecule could not transduce growth signal when its serine-rich region was deleted. However, the serine-rich regions in gp130 and IL-2-R β chain differed somewhat—i.e., the former includes 14 serines in the 30 successive amino acids (47%), but the latter does not include such a high serine frequency. In the serine-rich region of IL-2R β chain, a leucine residue, but not the serine residue itself, was critical for signal transduction (18). That the GTP-binding motif-like sequences in gp130 were not required for generating, at least, the growth signal may support the argument that the order of these four sequences and spacing between them differed from those in *ras* and *ras*-related proteins (4).

The 61-amino acid segment is significantly homologous with other members of the cytokine-receptor family. Although sequence similarities in the extracellular region of the cytokine-receptor family members are well documented (6–8), those in the cytoplasmic region have been little discussed. The cytoplasmic homologous part contains two highly conserved segments (boxes 1 and 2). These segments are not only structurally conserved but also are shown to be actually required for gp130 function: mutations in either of the segments abolished signal transduction. Interestingly, a previously identified amino acid in IL-2-R β chain important for the growth signal (the above-mentioned leucine; ref. 18) is located in box 2 (the latter L in this box; see Fig. 1*B*). Two of the nonfunctional gp130 mutant molecules, gp130PP and gp130IW, carry the amino acid substitution in box 1. Substitution of the two prolines by serines in the former mutant may have changed the tertiary structure of the cytoplasmic region of gp130 because proline is considered the helix-breaker and is thought to be important in protein structure (33). For example, the hinge region of immunoglobulins is rich in prolines, which may affect flexibility of the molecule and ability to bind complement (34). Substitution of the three conserved hydrophobic amino acids in box 1 (gp130IW) probably disturbed the hydrophobic interaction that may be required for folding of the important part of the cytoplasmic region. The carboxyl-terminal seven amino acids in box 2 are shown important for signal transduction. Further studies are necessary to know whether these amino acids, as well as the box 1 segment and the positively charged amino acids substituted in gp130POS, are cooperatively involved in folding of the important 61-amino acid segment or in association with downstream molecules.

The finding of two highly conserved segments in the homologous part of several receptors or receptor associate molecules raises the possibility that a common or structurally related signaling molecule(s) might be interacting with the cytoplasmic region of these membrane proteins belonging to the cytokine-receptor family. Examining this possibility may explain the functional redundancy of the cytokines. It would be of interest were the homologous part to play a role in the interaction of tyrosine kinases. Several cytokines, such as IL-2, IL-3, and IL-7, have been reported to induce tyrosine-phosphorylation of cellular proteins and activation of tyrosine kinases (30–32). In contrast, the growth factor receptors such as epidermal growth factor receptor, colony-stimulating factor 1 receptor, and platelet-derived growth factor receptor, which possess the tyrosine kinase domain in the cytoplasmic region, do not contain box 1- and box 2-like se-

quences. For gp130, we indicated that gp130 was tyrosine-phosphorylated upon stimulation with IL-6 plus sIL-6-R. Although direct interaction of tyrosine kinase with gp130 was not shown in BAF130 cells, the present study implies that tyrosine kinase may play a role in IL-6 signal transduction through gp130. The wild-type gp130 molecule was tyrosine-phosphorylated upon IL-6 stimulation of the cells, and loss of signal-transducing ability of gp130 coincided with disappearance of the tyrosine phosphorylation of gp130, when only two prolines in box 1 were substituted (see gp130PP in Fig. 5). A possible involvement of tyrosine kinase in the IL-6 signal-transduction pathway or a direct interaction of tyrosine kinase with gp130 has to be examined. Because the ≈60-amino acid segment of the cytoplasmic region of gp130, IL-2-R β chain, Epo-R, and IL-3-R are homologous, the signals through these molecules are suggested to be similar. In fact, these receptors all possess the ability to generate growth signal in BAFB03 cells (17, 19). However, the signaling mechanism through them differs. The parental BAFB03 cells and the IL-2-R β chain- or Epo-R-transfected cells could be grown long term in the presence of IL-3, IL-2, or erythropoietin, respectively (17–19). In contrast, IL-6 stimulation (i.e., a complex of IL-6 and sIL-6-R), even at a saturable concentration, could only support the transient growth of BAFB03 transfectants expressing gp130 (data not shown). Furthermore, transfection of murine IL-2-dependent CTLL2 cells with human gp130 cDNA did not lead the cells to the acquisition of IL-6 responsiveness (M.H., M. Saito, and M.M., unpublished data). One explanation for these observations is that a downstream signaling molecule interacting with the homologous region of each of the above receptors (including gp130) is similar but not identical.

We showed that the 61-amino acid segment of the cytoplasmic region of gp130 was sufficient for generating the IL-6-mediated growth signal. Because IL-6 is a typical pleiotropic cytokine, conceivably different parts of the cytoplasmic region of gp130 might be required for mediating the other IL-6 functions (1, 2), such as immunoglobulin production in B cells, macrophage differentiation of myeloid leukemic cells, and neural differentiation.

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Dimerization of Cell Surface Receptors in Signal Transduction

Review

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Introduction

Cell growth, differentiation, migration, and apoptosis are in part regulated by polypeptide growth factors or cytokines. As these factors are unable to pass the hydrophobic cell membrane, a fundamental question is how they transduce their signals into the cell. Growth factors and cytokines exert their effects via binding to cell surface receptors; results obtained during recent years have given ample evidence that such receptors often are activated by ligand-induced dimerization or oligomerization. Moreover, the elucidation of intracellular signal transduction pathways have revealed that the activity of several components in these pathways are also regulated by dimerization. For instance, certain of the cytoplasmic signal transduction molecules dimerize after activation, and the active form of transcription factors are often dimers. It thus appears that dimerization is a mechanism of general applicability for the regulation of signal transduction.

This review focuses on the role of dimerization of cell surface receptors in signal transduction. Dimerization or oligomerization have been shown to occur after binding of several polypeptide hormones, cytokines, growth factors, or growth inhibitors to their receptors. Examples include protein-tyrosine kinase receptors, cytokine receptors, antigen receptors, receptors for tumor necrosis factor (TNF) and related factors, and serine/threonine kinase receptors (Figure 1; Table 1). There are, however, many variations on the theme, as will be discussed below.

Protein-Tyrosine Kinase Receptors

Many traditional growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF), bind to receptors with tyrosine kinase activity (Table 1). Protein-tyrosine kinase receptors consist of single transmembrane domains separating the intracellular kinase domains from extracellular domains, which typically contain one or several copies of immunoglobulin-like domains, fibronectin type III–like domains, EGF-like domains, cysteine-rich domains, or other domains (reviewed by Fantl et al., 1993). Based on their structural characteristics, the tyrosine kinase receptors can be classified into families; the largest families are listed in Table 1.

Several of the ligands for protein-tyrosine kinase receptors are dimeric molecules, which thus contain two identical receptor-binding epitopes. Examples include PDGF and colony-stimulating factor 1 (CSF-1), which are disulfide-bonded dimers, and stem cell factor (SCF), which is a dimer held together by noncovalent forces. These ligands form stable receptor dimers by simultaneously binding two receptors. In addition to the bridging of the ligand between two receptors, it is possible that direct interactions between the receptors, involving epitopes located outside the ligand-binding domains, are important for stabilization of the receptor dimer. In the case of the SCF receptor, evidence has been presented that epitopes in the fourth immunoglobulin domain are involved in such receptor–receptor interactions (Blechman et al., 1995). It is possible that such direct receptor–receptor interactions are promoted by conformational changes in the receptors induced by ligand binding. Other ligands, like EGF, have apparent monomeric configurations; interestingly, however, recent calorimetric studies have shown that a single EGF molecule also can bind simultaneously to two receptor molecules (Lemmon and Schlessinger, 1994). Another variation on the theme is exemplified by ligands for Eph-related tyrosine kinase receptors. These ligands are cell surface attached and do not activate receptors in soluble form. The possibility that receptor dimerization or clustering is involved in receptor activation, presumably facilatated by membrane attachment of ligands, is supported by the finding that antibody-mediated clustering of soluble receptors led to activation of receptors (Davis et al., 1994).

Receptor Autophosphorylation

Dimerization of protein-tyrosine kinase receptors is followed by receptor "autophosphorylation," which mainly occurs by one receptor molecule phosphorylating the other in the dimer (Ullrich and Schlessinger, 1990). The autophosphorylation occurs on two principally different classes of tyrosine residues. On one hand, autophosphorylation is commonly seen on a conserved tyrosine residue within the kinase domains (Tyr-857 in the PDGF β receptor; Figure 2). In the cases of the receptors for insulin and hepatocyte growth factor (HGF), phosphorylation of the tyrosine residue at this and neighboring sites leads to an increase in the kinase activity and precedes phosphorylation of other sites in the receptor or substrates (Naldini et al., 1991; White et al., 1988). This thus appears to be an allosteric site that regulates the V_{max} of the receptor kinase. It is still not known how the autophosphorylation is initiated; one possibility is that the monomeric receptor has a low basal kinase activity, which is sufficient to phosphorylate and activate the companion receptor after dimerization. This would then rapidly be followed by reciprocal phosphorylation. Alternatively, the interaction between the intracellular domains of the receptors in the dimer may induce a conformational change that leads to an increased kinase activity. Not all receptors are regulated by phosphorylation inside the kinase domain, e.g., in the EGF receptor, the conserved tyrosine residue in the kinase domain appears not to be autophosphorylated.

The other class of autophosphorylation sites are normally localized outside the kinase domains and serve the important function of creating docking sites for downstream signal transduction molecules containing Src-homology 2 (SH2) domains. The SH2 domains consists of about 100 amino acid residues folded in such a way

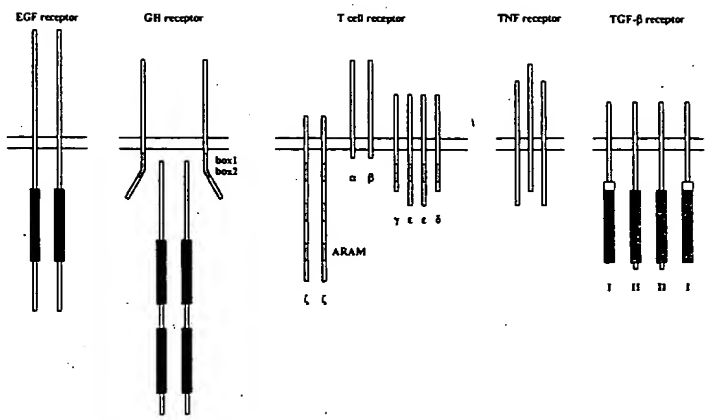

Figure 1. Examples of Receptors Activated by Dimerization or Oligomerization

Schematic representations of the complexes formed after ligand binding to receptors from the families discussed in the text, i.e., protein-tyrosine kinase receptors (the EGF receptor shown as an example), cytokine receptors (GH receptor bound to JAK kinases), antigen receptors (TCR), trimeric receptors (TNF receptor), and serine/threonine kinase receptors (TGFβ receptor). Tyrosine kinase domains are closed and serine/threonine kinase domains dark stippled. Box1, box2 (light stippled), refers to a region in cytokine receptors to which JAK kinases bind. ARAM (light stippled) refers to antigen recognition activation motifs in different subunits of antigen receptors that become phosphorylated by tyrosine kinases of the Src family and thereafter bind tyrosine kinases of the ZAP-70/Syk family.

Table 1. Families of Receptors Activated by Dimerization or Oligomerization

Receptor Type	Family	Examples	Characteristics
Protein-tyrosine kinase receptors	PDGF receptor family	PDGFR-α, PDGFR-β, SCFR (Kit), CSF-R (Fms), Flk-2	Five immunoglobulin-like domains extracellularly
	EGF receptor family	EGFR (ErbB), ErbB2 (Neu), ErbB3, ErbB4	Two cysteine-rich domains extracellularly
	FGF receptor family	FGFR-1, FGFR-2, FGFR-3, FGFR-4	Two to three immunoglobulin-like domains extracellularly
	IGF receptor family	insulin R, IGF-1R	Disulphide-bound heterotetramer of α and β chains
	HGF receptor family	HGFR (Met), MSPR (Ron)	Extracellular domain cleaved into an α and β chain
	VEGF receptor family	Flt-1, Flk-1 (KDR)	Seven immunoglobulin-like domains extracellularly
	Neurotrophin receptor family	Trk, TrkB, TrkC	
	Eph receptor family	Eph, Elk, Eck, Cck5, Sek, Eck, Erk	Two FNIII-like domains and a cysteine-rich domain extracellularly
Cytokine receptors	Class I cytokine receptor family		
	GH receptor subfamily	GHR, EPOR, PRLR, G-CSFR	Form homodimers
	IL-3 receptor subfamily	IL-3R, GM-CSFR, IL-5R	Form complexes with the β_c subunit
	IL-6 receptor subfamily	IL-6R, LIFR, CNTFR, IL-11R	Form complexes with gp130
	IL-2 receptor subfamily	IL-2Rα, IL-2rβ, IL-4R, IL-7R	Form complexes with IL-2Rγ
	Class II cytokine receptor family	IFN-α/βR, IFN-γRα, IFN-γRβ, IL-10R	
TNF receptor family		TNFR-1, TNFR-II, LNGFR, CD40, OX-40, Fas, CD27, CD30	Form trimers
Antigen receptors		TCR	Complex of α, β, γ, δ, ε, ζ an η subunits
		BCR	Complex of IgM and heterodimers of α/β subunits
Serine/threonine kinase receptor family	Type II receptor family	TGFβR-II, ActR-II, ActR-IIB	Form hetero-oligomers with type I receptors, i.e., TGFβR-I, ActR-1, ActR-1B, BMPR-IA, BMPR-IB, ALK-1

Receptor families and subfamilies discussed in the text are presented. Abbreviations used: R, receptor; PDGF, platelet-derived growth factor; SCF, stem cell factor; CSF, colony-stimulating factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; HGF, hepatocyte growth factor; MSP, macrophage-stimulating protein; VEGF, vascular endothelial growth factor; FN, fibronectin; GH, growth hormone; EPO, erythropoietin; PRL, prolactin; IL, interleukin; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor; IFN, interferon; TNF, tumor necrosis factor; LNGFR, low affinity nerve growth factor receptor; TCR, T cell receptor; BCR, B cell receptor; TGFβ, transforming growth factor β; Act, activin; BMP, bone morphogenic protein. Alternative designations are given within parentheses.

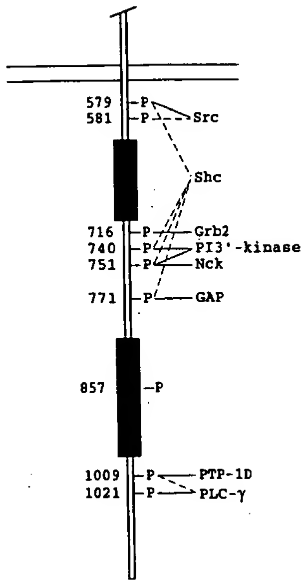

Figure 2. Interaction of SH2 Domain–Containing Signal Transduction Molecules with Different Autophosphorylation Sites in the PDGF β Receptor

Schematic illustration of the intracellular portion of a PDGF β receptor after activation. The kinase domain (closed boxes) in the receptor is divided into two parts by an inserted sequence. The tyrosine residues in the receptor known to be autophosphorylated are indicated by numbers. The interaction of individual autophosphorylated tyrosine residues with different SH2 domain–containing proteins are also indicated. Shc, Grb2, and Nck are adaptor molecules; Src denotes different members of the Src family of tyrosine kinases; PI3-kinase, phosphatidylinositol 3′-kinase; GAP, GTPase-activating protein; PTP1D, protein tyrosine phosphatase 1D; PLC-γ, phospholipase C-γ.

that a binding pocket for a phosphorylated tyrosine and the immediately surrounding amino acid residues is formed (Pawson and Schlessinger, 1993; Cohen et al., 1995 [this issue of *Cell*]). Of particular importance are the three to six amino acid residues C-terminal of the phosphorylated tyrosine (Eck et al., 1993; Pascal et al., 1994; Waksman et al., 1993); since different SH2 domains have different preferences for this region, there is specificity in the interaction. As an example, the PDGF β receptor has been shown to contain at least nine autophosphorylated tyrosine residues; Tyr-857 in the second part of the kinase domain is of importance for the catalytic activity of the kinase, whereas the others interact in a specific manner with at least eight different signal transduction molecules (reviewed by Claesson-Welsh, 1994; Figure 2).

Homodimerization or Heterodimerization

Protein-tyrosine kinase receptors are activated after homodimerization or after heterodimerization. In the case of the PDGF receptor subfamily, the different isoforms of PDGF induce different dimeric forms of the receptors. Since the A chain of PDGF binds only α receptors while the B chain binds both α and β receptors with high affinity, PDGF-AA induces αα receptor homodimers only, PDGF-AB induces αα receptor homodimers and αβ receptor heterodimers, and PDGF-BB induces all three combinations of receptors (Heldin et al., 1989; Kanakaraj et al., 1991; Seifert et al., 1989). There are certain differences in the signals transduced via αα receptor homodimers and ββ receptor homodimers, e.g., regarding the stimulation of chemotaxis and actin reorganization. Moreover, PDGF-AB, which preferentially induces αβ receptor dimers, induces a stronger mitogenic response than the other PDGF isoforms. A possible explanation for the unique properties of the αβ receptor heterodimer is the presence of unique autophosphorylation sites, not seen in the homodimeric receptors, and that may mediate interactions with additional signal transduction molecules (Rupp et al., 1994). Thus, the response to PDGF depends both on the particular isoform of PDGF and on the number of α and β receptors expressed on the target cells.

The EGF receptor was the first protein-tyrosine kinase receptor to be shown to dimerize after ligand binding (Yarden and Schlessinger, 1987). However, within the same subfamily of tyrosine kinase receptors, heterodimerization of receptors has also been observed. A candidate ligand for ErbB2 (Neu differentiation factor [NDF], also called heregulin, glial growth factor, or acetylcholin-receptor-inducing activity), which is structurally related to EGF, was found to induce heterodimeric complexes between ErbB2 and ErbB3 or ErbB4 (Peles et al., 1993; Plowman et al., 1993; Sliwkowski et al., 1994). Moreover, the presence of ErbB3 or ErbB4 was necessary for high affinity binding of NDF and signal transduction through ErbB2 to occur. Interestingly, ErbB3 lacks certain highly conserved amino acid residues in its kinase domain; consistent with this finding, ErbB3 was found to have low or no kinase activity (Prigent and Gullick, 1994). It is thus possible that the major function of ErbB3 in the heterodimer is to act as a substrate for the ErbB2 kinase and thus provide docking sites for downstream SH2 domain–containing signal transduction molecules (Carraway and Cantley, 1994); for example, binding motifs for the SH2 domains of the phosphatidylinositol 3′-kinase (PI3-kinase) are lacking in the EGF receptor and in ErbB2, but occur in several copies in ErbB3 (Fedi et al., 1994; Soltoff et al., 1994). Also, EGF itself can induce heterodimerization of EGF receptors and ErbB2 (Soltoff et al., 1994; Wada et al., 1990). In fact, heterodimerization is preferred in cells expressing both EGF receptors and ErbB2. Although heterodimerization occurred also with a kinase-inactivated ErbB2 receptor mutant, this complex was inactive, showing that in this case signaling can not occur via ErbB2 serving as a EGF receptor substrate (Qian et al., 1994).

The studies on dimerization of receptors in the PDGF receptor and EGF receptor families thus provide examples of different types of dimeric complexes induced after ligand binding, i.e., homodimeric (Figure 3A) or heterodimeric (Figure 3B) complexes between two catalytically active subunits, or a heterodimeric complex between one active and one inactive or less active subunit (Figure 3C). Given that tyrosine kinase receptors and ligands occur in families of structurally related molecules, it is not unlikely that homodimerization and heterodimerization of receptors occur in parallel also in other families, thus increasing

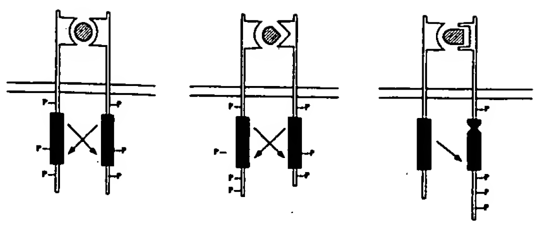

Figure 3. Different Dimeric Complexes of Protein-Tyrosine Kinase Receptors

Schematic representation of different forms of dimeric complexes of tyrosine kinase receptors formed after ligand binding. (A) a homodimeric complex; (B) a heterodimeric complex of two kinase-active subunits; (C) a heterodimeric complex of one active and one inactive or less active subunit.

the range of responses possible from a given number of receptor molecules.

One special case is the insulin and insulin-like growth factor 1 (IGF-1) receptor family. These receptors exist in the cell membrane as disulfide-bonded homo- or heterodimers of receptor subunits (each subunit is further cleaved into α and β chains by proteolysis) (Soos and Siddle, 1989). Thus, ligand binding does not induce receptor dimerization, but presumably causes a conformational alteration in the preformed dimeric receptor, which leads to receptor activation. Moreover, autophosphorylated tyrosine residues in the receptor molecules are not so important for the binding of downstream components in the signal transduction pathways; rather, the insulin receptor kinase phosphorylates insulin receptor substrate 1 (IRS-1), which mediates the interactions with SH2 domain proteins (White, 1994).

Cytokine Receptors

The cytokine receptor classes include receptors for many interleukins, colony-stimulating factors, interferons, and certain other factors and hormones (reviewed by Kishimoto et al., 1994; Mui and Miyajima, 1994; see Table 1). Class I cytokine receptors are characterized by the presence in their extracellular domains of one or two copies of a conserved domain of about 200 amino acids, which contains two modules of fibronectin type III-like motifs, four conserved cysteine residues, and the conserved motif Trp–Ser–Xaa–Trp–Ser (Bazan, 1990). Class II cysteine receptors, including receptors for interferons and interleukin-10 (IL-10), contain another conserved motif of four cysteine residues and lack the Trp–Ser–Xaa–Trp–Ser motif. The intracellular domains of cytokine receptors lack intrinsic enzymatic activities. However, despite the structural difference between cytokine receptors and tyrosine kinase receptors, their mechanism of activation appears to be similar. Ligand binding induces dimerization or oligomerization of cytokine receptors, and this allows interaction and activation of cytoplasmic protein-tyrosine kinases that are associated with the intracellular domain of the receptors.

Activation of Class I Cytokine Receptors through Formation of Hetero-Oligomeric Complexes

Most of the class I cytokine receptors undergo heterodimerization or hetero-oligomerization after ligand binding (Table 1). In many cases, the ligand-binding subunit(s) form signaling complexes with signal-transducing molecules that are structurally related to cytokine receptors, but that are themselves unable to bind ligands. For instance IL-3, granulocyte/macrophage colony-stimulating factor (GM-CSF), and IL-5 bind to specific α subunit receptors; the α subunits all interact with a common β subunit that is required for high affinity ligand binding and signal transduction (Mui and Miyajima, 1994).

Similarly, IL-6, leukemia inhibitory factor (LIF), oncostatin M, IL-11, and ciliary neurotrophic factor (CNTF) share a common signal transducer, gp130 (Taga et al., 1989); signaling is triggered by the formation of homo- or heterodimers of gp130. IL-6 binds to the IL-6 receptor and induces a complex containing a homodimer of gp130 (Murakami et al., 1993). Interestingly, signaling occurs also with a truncated IL-6 receptor lacking the cytoplasmic domain, which indicates that the IL-6 receptor is needed only to increase the binding affinty for IL-6. The CNTF receptor acts similarly, lacking a cytoplasmic domain in its natural form and being anchored in the membrane through a phosphatidylinositol group. The CNTF receptor–CNTF complex signals via formation of a heteromeric complex of gp130 and the LIF receptor (Davis et al., 1993). LIF and oncostatin M signal via binding directly to a heteromeric complex of gp130 and the LIF receptor (Gearing et al., 1992). IL-11 is dependent on gp130 but not the LIF receptor for signaling (Hilton et al., 1994).

A third subfamily is constituted by IL-2, IL-4, IL-7, and IL-9. In this family, signaling involves the formation of heterodimeric receptor complexes between specific β subunits and a common γ subunit (Kawahara et al., 1994). In the case of IL-2, the ligand binding affinity is increased by the presence also of an α subunit, which has a structure unrelated to that of cytokine receptors. Whereas the α subunit is not needed for signal transduction, both the β and the γ subunits are needed, presumably in a heterodimeric configuration (Nakamura et al., 1994; Nelson et al., 1994).

Activation by Homodimerization

Although activation by heterodimerization appears to be most common among cytokine receptors, there are examples of cytokine receptors that are activated by homodimerization, e.g., the receptors for growth hormone (GH), erythropoietin (EPO), prolactin, and granulocyte colony-stimulating factor (G-CSF) (Table 1). A well-characterized example is the GH receptor. Analysis of crystals of GH and the extracellular part of the receptor revealed that each ligand binds two receptor molecules simultaneously (Cunningham et al., 1991; de Vos et al., 1992; Ultsch et al., 1991). This finding was surprising since GH is a monomeric molecule without apparent symmetry. The two receptor-binding sites in GH are therefore different, although they bind to similar epitopes in the receptors. Site 1 is larger and is supposed to bind receptor first; the smaller site 2 thereafter binds a second receptor, and the dimeric receptor complex is further stabilized by direct interaction between the two receptors. The importance of the latter

epitope in stabilizing a GH receptor dimer is illustrated by the finding that a mutation in this region abolishes receptor homodimerization and is responsible for a form of familial GH resistance (Laron's syndrome; Duquesnoy et al., 1994). The results from the three-dimensional structure studies are supported by titration calorimetry in solution; the heat of binding was found to be saturated at a 1:2 ratio of ligand and receptor (Ultsch et al., 1991).

Signal Transduction

Much information regarding the signal transduction pathways from cytokine receptors to the nucleus has come from a genetic approach in which mutant cell lines defective in the response to interferons were isolated and characterized (Darnell et al., 1994). This approach led to the identification of three categories of proteins, a DNA-binding protein (p48), STATs (signal transducers and activators of transcription), and cytoplasmic protein-tyrosine kinases of the JAK family.

The JAK kinases are characterized by the presence of two kinase domains in each molecule, which is the basis for their name (Janus kinases, after the Roman god with two faces) (reviewed by Ziemiecki et al., 1994). Several members of this family are currently known (JAK1, JAK2, Tyk2, JAK3 [Ziemiecki et al., 1994; Takahashi and Shirasawa, 1994]); they associate in a specific manner with different cytokine receptors and are activated upon receptor dimerization. An important class of substrates for JAK kinases is members of the STAT family (STAT1α, STAT1β, STAT2, STAT3, STAT4, STAT5, and IL-4 STAT) (Darnell et al., 1994; Zhong et al., 1994; Gouilleux et al., 1994; Hou et al., 1994). After phosphorylation on tyrosine residues, the STAT molecules form homo- or heterodimers.

In the case of signaling from the interferon-α receptor, heterodimers of STAT1α (p91) or STAT1β (p84) and STAT2 (p113) are created, which move into the nucleus and form a complex with a DNA-binding protein (p48), allowing them to bind and stimulate transcription from elements in the promoters of interferon-α-induced genes (Schindler et al., 1992). Interestingly, another combination of STATs is formed after stimulation by interferon-γ, either a homodimer of STAT1α (p91) or a homodimer of STAT1β (p84) (Shuai et al., 1994; Shuai et al., 1992). These dimers do not associate with p48, but may form complexes with other related proteins. Both homodimers bind to interferon-γ-activated sites (GAS), which are present in interferon-γ-inducible genes, although only the STAT1α homodimer activates transcription (Shuai et al., 1993).

It thus appears that the activities of STATs are regulated by specific assembly into homo- or heterodimers. The dimerization is triggered by phosphorylation. A single phosphorylated tyrosine residue has been identified in STAT1 after stimulation with interferon-α as well as after stimulation with interferon-γ; mutation of this tyrosine residue to a phenylalanine residue prevents dimerization (Shuai et al., 1993). Since STATs contain SH2 domains, it is likely that the dimerization involves reciprocal interactions between the SH2 domains and the tyrosine-phosphorylated regions in the STAT molecules (Shuai et al., 1994). How is the specificity regulated? One possibility is that STATs may associate in a differential manner with different receptors (Fu and Zhang, 1993; Greenlund et al., 1994).

Another possibility is that the JAK family members differ in their substrate specificities and thus phosphorylate different STAT molecules. Interestingly, the genetic approach led to the identification of different JAK kinases in the signaling pathways of interferon-α (JAK1 and Tyk2) and interferon-γ (JAK1 and JAK2) (Müller et al., 1993; Velazques et al., 1992; Watling et al., 1993). Thus, in each case, there was a need for two different JAK kinases. It is unlikely that the two kinases are needed in a sequential activation mechanism, since in cells deficient in JAK1 no activation of JAK2 was seen after stimulation with interferon-γ, or vice versa (Müller et al., 1993). Thus, it is possible that the active forms of the JAK kinases involved in the signal pathways of interferons are activated by heteromeric interactions, possibly involving cross-phosphorylations.

A possible mechanism to achieve such heterodimerization is via ligand-dependent formation of heteromeric receptor complexes. The receptor for interferon-γ consists of at least two different chains (Aguet et al., 1988; Hemmi et al., 1994; Soh et al., 1994), and it has been suggested that JAK1 and JAK2 interact with these chains in a differential manner (Greenlund et al., 1994). An interferon-α/β receptor that binds JAK1 has been identified (Novick et al., 1994); whether another receptor subunit with affinity for Tyk2 exists remains to be elucidated. An analogous situation appears to prevail for the IL-2 receptors; the β and γ subunits have been shown to bind JAK1 and JAK3, respectively (Miyazaki et al., 1994; Russell et al., 1994).

Common and Unique Signals

In addition to the receptors for interferon-α and interferon-γ, many other receptors, including GH, EPO, prolactin, G-CSF, LIF, gp130, the common β subunit for the IL-3 subfamily of receptors, and the common γ subunit for the IL-2 subfamily, have been shown to bind different members of the JAK family (Ihle et al., 1994). JAKs bind in a specific manner to conserved regions called box 1 and box 2 regions in the juxtamembrane parts of cytokine receptors (Murakami et al., 1991).

Other signal transduction pathways are also initiated at the activated cytokine receptor complexes; these pathways are dependent on more C-terminal regions in the receptors. For instance, members of the Src family of kinases bind to the C-terminal tail of the IL-2 β receptor (Hatakeyama et al., 1991) and to gp130 (Ernst et al., 1994). Moreover, whereas JAK kinases bind to the juxtamembrane part of the common β subunit of IL-3, IL-5, and GM-CSF, deletion of the C-terminus abrogates Shc phosphorylation, Ras activation, and induction of *c-fos* and *c-jun* (Sato et al., 1993). Likewise, a region C-terminal of the JAK kinase–binding site of the G-CSF receptor mediates induction of granulocyte-specific genes (Fukunaga et al., 1993).

The fact that certain receptor subunits/signal transducers are shared by several cytokines, as well as the fact that different receptors may bind and activate the same JAK kinases and possibly also share other signal transduc-

tion molecules, provides an explanation for the functional redundancy and pleiotropy of different cytokines. Conversely, the presence of unique epitopes in receptors or receptor combinations may allow the transduction of specific signals that mediate unique properties of the different cytokines.

Sharing of Signal Transduction Pathways between Tyrosine Kinase Receptors and Cytokine Receptors

There is no sharp division in the modes of signaling between tyrosine kinase receptors and cytokine receptors. EGF and PDGF, acting via tyrosine kinase receptors, induce the phosphorylation of STAT1α, perhaps directly or via activation of JAK kinases (Fu and Zhang, 1993; Ruff-Jamison et al., 1993; Sadowski et al., 1993; Silvennoinen et al., 1993). Moreover, after activation of cytokine receptors, JAK kinases or possibly other kinases phosphorylate the cytokine receptors themselves. This gives SH2 domain–containing signal transduction molecules the possibility to interact with the cytokine receptors and initiate pathways initially identified for tyrosine kinase receptors, e.g., leading to activation of Ras and PI3-kinase (Boulton et al., 1994; Mui and Miyajima, 1994).

Antigen Receptors

The T cell receptor is composed of transmembrane proteins with very short cytoplasmic sequences, which are associated with a large number of invariant subunits also lacking intrinsic enzymatic activities, but capable of interacting with cytoplasmic tyrosine kinases (Figure 1) (for reviews see Cambier and Jensen, 1994; Weiss and Littman, 1994). The invariant subunits (γ, δ, ϵ, ζ, and η chains) contain one to three copies of a conserved 26 amino acid motif of pairs of tyrosine and leucine residues (Reth, 1989), called the antigen recognition activation motif (ARAM; also called tyrosine-based activation motif, or antigen receptor homology 1). Tyrosine kinases of the Src family bind to the T cell receptor even in the resting state. In conjunction with receptor activation, the tyrosine residues in the ARAMs are phosphorylated, presumably by Src family kinases (in T cells primarily Lck). This gives another tyrosine kinase, ZAP-70, which has two SH2 domains, the opportunity to bind to the phosphorylated sites, after which it becomes activated by phosphorylation on tyrosine residues, most likely also by Src family tyrosine kinases (Iwashima et al., 1994; Letourneur and Klausner, 1992). The precise mechanism that triggers antigen receptor activation and phosphorylation of the ARAM sequences is not known, although a possible scenario is that antigen binding causes receptor aggregation that makes possible interactions and cross-phosphorylation of tyrosine kinases in the Src family. Consistent with this possibility are the observations that chimeric molecules consisting of cytoplasmic parts of ARAM–containing T cell receptor subunits and extracellular domains of other molecules mediate activation of T cells after cross-linking (Letourneur and Klausner, 1992; Irving and Weiss, 1991; Romeo and Seed, 1991).

The B cell receptor and Fc receptors also occur in complexes containing signal transducing molecules with ARAMs, suggesting similar mechanisms of signal transduction (Clark et al., 1994; Law et al., 1993; Ravetch, 1994). Interestingly, a sequential activation of Src family members and ZAP-70/Syk family members may also be involved in cytokine signaling. The G-CSF receptor has been shown to be associated with Lyn, a member of the Src family; after stimulation, an ARAM-like motif in the C-terminus of the G-CSF receptor is phosphorylated, which binds Syk leading to its activation (Corey et al., 1994).

TNF Receptor Family

An interesting variation on the "activation by oligomerization" theme is provided by members of the TNF receptor family, which are involved in regulation of cytotoxicity, apoptosis, and proliferation (for reviews see Bazan, 1993; Smith et al., 1994). TNF occurs as two forms, TNFα and TNFβ, which both binds to two different receptors, TNF receptor 1 and 2 (p55 and p75, respectively). The TNFs are nondisulfide-bonded trimers, and elucidation of the X-ray structure of TNFβ and TNF receptor 1 (Banner et al., 1993) revealed that ligand binding induces trimerization of the receptor. Each TNF subunit makes contact with two adjacent receptor molecules, thus stabilizing the receptor trimer. It is likely that the activating event is receptor aggregation, but it is not clear whether there is a need for receptor trimerization, or whether receptor dimerization would be sufficient for activation. In support of the possibility that trimerization of TNF receptor 1 is, in fact, necessary for signal transduction, monoclonal antibodies against this receptor, which are expected to dimerize the receptor, do not lead to activation, whereas activation occurs after cross-linking of the monoclonals with a second antibody, or after stimulation by two monoclonals directed against different epitopes (Engelmann et al., 1990).

A novel family of molecules that associate with the cytoplasmic part of TNF receptor 2 and that may serve as signal transducers was recently identified (Rothe et al., 1994); TNF receptor associated factors, TRAF1 and TRAF2, contain a novel region of homology and form homo- or heterodimers. This finding represents an important step in the understanding of signaling from the TNF receptor 2, but the mode of activation of TRAFs, their downstream effectors, and whether related molecules are involved in signaling from other members in the TNF receptor family remain to be elucidated.

Protein-Serine/Threonine Kinase Receptors

Transforming growth factor β (TGFβ) is a prototype for a large family of structurally related factors that regulate cell growth and differentiation, including in addition to TGFβs, e.g., activins and inhibins, bone morphogenic proteins, and Müllerian inhibition substance. As far as has been characterized, these molecules exert their cellular effects by binding to heteromeric complexes of serine/threonine kinase receptors (reviewed by Massagué et al., 1994; Miyazono et al., 1994).

Both type I and type II receptors have rather small cysteine-rich extracellular domains; the type I receptors, which are more similar to each other than to the type II receptors, all have a characteristic region rich in glycine and serine

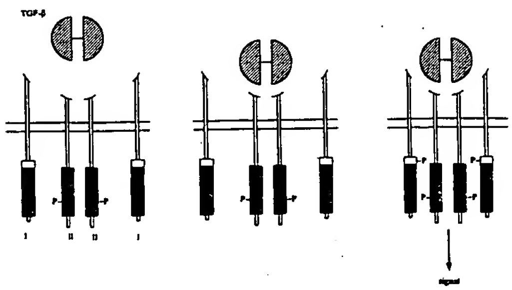

Figure 4. Signaling via TGFβ Receptors

Schematic illustration of the mechanism of signaling via TGFβ receptors as suggested by Wrana et al. (1994). TGFβ binds first to type II receptors that have a constitutively active serine/threonine kinase. The type I receptor is then incorporated in the complex and activated by phosphorylation in the GS box. Serine/threonine kinase domains are stippled, and GS boxes are open.

residues (GS domain) in their cytoplasmic juxtamembrane domains. Both receptors are needed for signaling (Wrana et al., 1992), and the cytoplasmic parts of the receptors are not interchangeable (Okadome et al., 1994). Ligand binding induces a hetero-oligomeric complex of type I and type II receptors, most likely a heterotetramer containing two receptors of each type (Yamashita et al., 1994). Studies on TGFβ-induced phosphorylation of the receptor molecules have led to an interesting model for activation of the receptors (Wrana et al., 1994; Figure 4). The type II receptor, which occurs in a dimer also in the absence of ligand (Henis et al., 1994; Chen and Derynck, 1994) and has a constitutively active kinase, first binds TGFβ. This complex then recruits the type I receptor, which can not bind ligand in the absence of type II receptor, resulting in the phosphorylation of the type I receptor on serine residues in the GS domain. The phosphorylation presumably activates the type I receptor kinase that now can act on downstream components in the signal transduction pathway. Other members in the TGFβ family also form heteromeric complexes containing different members of the type I and type II receptor subfamilies. Thus, sequential phosphorylation between the type II and type I receptors may be a general mechanism of receptor activation of members of the serine/threonine kinase receptor family.

Is Dimerization Sufficient for Activation?

There are several examples in which activation of receptors occurs after dimerization or oligomerization induced by means other than ligand binding. Many tyrosine kinase receptors, for instance, are activated after binding of antibodies, whereas Fab fragments generally are inactive. Insertion of an extra cysteine residue in the extracellular juxtamembrane region of the EGF receptor led to the formation of a constitutively active dimeric receptor (Sorokin et al., 1994). Moreover, mutated forms of many of the tyrosine kinase receptors have been identified as transforming oncogenes. In some cases, the activating mechanism is a gene rearrangement that leads to the production of a fusion protein between a novel protein and the kinase domain of the receptor. The fusion partners are often domains of proteins that undergo oligomerization in their normal context. Examples include tropomyosin, which has been found fused to Trk (Martin-Zanca et al., 1986), the regulatory subunit of the cyclic AMP-dependent protein kinase, which has been found fused to Ret (Takahashi et al., 1985), and sequences from Tpr, containing a leucine zipper, which has been found fused to Met (Park et al., 1986; Rodrigues and Park, 1994) as well as to Trk (Greco et al., 1992). Another mechanism is exemplified by the Neu (ErbB2) oncogene product, which obtained transforming activity by a single amino acid exchange in the transmembrane region that promotes receptor aggregation (Weiner et al., 1989). In these cases, artificially induced receptor dimerization leads to activation of the kinase domains and autophosphorylation in a ligand-independent manner.

Also cytokine receptors can acquire transforming properties after mutation. A constitutively active EPO receptor mutant was found to have an arginine residue replaced with a cysteine residue in a region corresponding to the receptor dimer interface of the related GH receptor; this resulted in the formation of a disulfide bond that stabilized the receptor dimer in a ligand-independent manner (Watowich et al., 1992). This finding further supports the concept that dimerization is sufficient for activation of many receptor types.

Antagonists

There are many examples of tyrosine kinase receptors and cytokine receptors that after mutations in their cytoplasmic domains act in a dominant negative manner, i.e., when expressed in cells with the corresponding wild-type receptor, they attenuate the signals induced by ligands. The mechanism for the dominant negative effect is that the wild-type receptors after ligand binding are locked up in sterile heteromeric complexes with the mutated receptors. These findings provide support for the notion that dimerization of wild-type receptors is necessary for activation of many receptor types; however, alternative modes for activation have not been excluded. Another way in which the oligomerization process can be antagonized is through mutated versions of certain ligands. For example, mutation of one of the two receptor-binding sites in GH yielded a GH protein with antagonistic properties (Fuh et al., 1992). Moreover, mutation of a glutamic acid residue in GM-CSF (Glu-21) that is important for the interaction with the common β subunit (Hercus et al., 1994), mutation of Tyr-124 in IL-4, which is important for interaction with the common γ subunit (Kruse et al., 1992), or mutation of Tyr-31 and Gly-35 in IL-6, which are important for interaction with the gp130 signal transducer (Savino et al., 1994), yielded molecules with antagonistic effects in their respective systems.

It is possible that inhibition of receptor oligomerization is a generally applicable method to antagonize growth factor and cytokine action. Antagonistic ligands and antibodies may have particular clinical utility in conditions of overactivity of growth factors and cytokines, since they can act specifically.

Conclusions

It is now well established that several receptor types are activated through ligand-induced receptor dimerization or oligomerization. Dimerization combines accuracy with flexibility; there is specificity in binding of the ligand to the receptors and flexibility in the assembly of different homo- or heterodimeric receptor subunits depending on which receptors and signal transducers are expressed by a particular cell. There are also examples of receptors that do not dimerize after ligand binding, e.g., the serpentine receptor family, which transverses the cell membrane seven times and couples to G proteins, and ion channel receptors. However, for receptor molecules that are anchored in the membrane with a single transmembrane domain, dimerization or oligomerization may be a general mechanism for receptor activation.

A general feature of receptors generating growth stimulatory signals seems to be activation of tyrosine kinases in the receptor complex. Although the exact mechanisms for activation of the kinases remain to be elucidated, interactions and cross-phosphorylations between identical or related kinases induced by receptor dimerization are common. The resulting phosphorylations of tyrosine residues on receptor and signal transducing components trigger interactions with SH2-containing molecules (see Cohen et al., 1995). Growth inhibitory signals from the activated TGFβ receptor complex involve phosphorylation on serine/threonine residues in yet unknown substrates. Thus, much of intracellular signaling is regulated by phosphorylation events. To understand the regulation of signal transduction, it will therefore be important to characterize not only the kinases involved, but also the phosphatases that counteract the effects of kinases (see Hunter, 1995 [this issue of *Cell*]).

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Point mutations within a dimer interface homology domain of c-Mpl induce constitutive receptor activity and tumorigenicity

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c-Mpl, a receptor for thrombopoietin (TPO), belongs to the haemopoietin/cytokine receptor superfamily, a group of cell surface molecules characterized by conserved sequence motifs within their ligand binding domains. A recurring mechanism for the activation of haemopoietin receptors is the formation of functional complexes by receptor subunit oligomerization. Within the growth hormone receptor, a cluster of extracellular amino acids forms a dimer interface domain that stabilizes ligand-induced homodimers. This domain appears to be functionally conserved in the erythropoietin (EPO) receptor because substitution of cysteines for residues in the analogous region causes EPO-independent receptor activation via disulfide-linked homodimerization. This report identifies an homologous domain within the c-Mpl receptor. The substitution of cysteine residues for specific amino acids in the dimer interface homology regions of c-Mpl induced constitutive receptor activity. Factor-dependent FDC-P1 and Ba/F3 cells expressing the active receptor mutants no longer required exogenous factors and proliferated autonomously. The results imply that the normal process of TPO-stimulated Mpl activation occurs through receptor homodimerization and is mediated by a conserved haemopoietin receptor dimer interface domain. Moreover, cells expressing activated mutant Mpl receptors were tumorigenic in transplanted mice. Thus, like v-*mpl*, its viral counterpart, mutated forms of the cellular *mpl* gene also have oncogenic potential.

Keywords: c-*mpl*/haemopoietin receptor/tumorigenicity

Introduction

The c-*mpl* gene was discovered as the cellular homologue of v-*mpl*, the oncogene of the murine myeloproliferative leukaemia virus (MPLV; Souryi *et al.*, 1990; Vigon *et al.*, 1992). In mice, v-*mpl* induces a lethal myeloproliferative disease characterized by the rapid appearance of factor-independent haemopoietic progenitors and an acute leukaemia involving multiple haemopoietic lineages (Wendling *et al.*, 1986, 1989). The cellular gene encodes a cell surface receptor that is expressed predominantly in primitive haemopoietic cells, megakaryocytes and platelets (Souryi *et al.*, 1990; Vigon *et al.*, 1992; Methia *et al.*, 1993; Debili *et al.*, 1995). Consistent with this expression pattern, the ligand for c-Mpl has been identified recently as thrombopoietin (TPO, also referred to as Mpl ligand or megakaryocyte growth and development factor; Bartley *et al.*, 1994; de Sauvage *et al.*, 1994; Lok *et al.*, 1994). In culture, recombinant TPO stimulates the proliferation of megakaryocyte progenitors (CFU-Mk) and induces maturation of megakaryocytes. Its administration to mice also stimulates CFU-Mk production, elevates megakaryocyte numbers in the bone marrow and spleen and significantly increases the number of circulating platelets (de Sauvage *et al.*, 1994; Kaushansky *et al.*, 1994; Broudy *et al.*, 1995). The c-Mpl receptor is clearly essential for normal thrombopoiesis. Reagents that neutralize TPO activity prevent normal megakaryocyte development *in vitro* (Wendling *et al.*, 1994; Kaushansky *et al.*, 1995), and mice in which c-*mpl* has been disrupted via homologous recombination in ES cells produce only 10–20% of the normal number of megakaryocytes and platelets (Gurney *et al.*, 1994).

c-Mpl belongs to the haemopoietin or cytokine receptor superfamily, members of which share a conserved extracellular haemopoietin receptor domain defined by four evenly spaced cysteine residues, series of alternating hydrophobic and polar residues and the distinctive Trp–Ser–Xaa–Trp–Ser (WSXWS) motif (Gearing *et al.*, 1989; Bazan, 1990; Cosman *et al.*, 1990). A recurring theme within this family is receptor subunit oligomerization, which characterizes the process of ligand-stimulated receptor activation. Many haemopoietin receptors form active, high affinity complexes through the hetero-oligomerization of ligand-specific α-chains with shared signal transducing β-chains. Interleukin (IL)-2, IL-4, IL-7, IL-9 and IL-13 each bind a specific receptor chain and recruit a common component, the IL-2 receptor γ-chain, to the active complex (reviewed in Taniguchi and Minami, 1993; Kishimoto *et al.*, 1994). Similarly, IL-3, IL-5 and granulocyte–macrophage colony-stimulating factor bind specific receptors at low affinity and then interact with the shared β_c chain at high affinity for signal transduction (reviewed in Nicola and Metcalf, 1991; Miyajima *et al.*, 1993). Finally, the receptors for IL-6, IL-11, leukaemia inhibitory factor (LIF), oncostatin M and ciliary neurotropic factor form active complexes which combine α-chains with the LIF receptor and/or gp130 (Hibi *et al.*, 1990; Gearing *et al.*, 1992; Ip *et al.*, 1992; Davis *et al.*, 1993; Hilton *et al.*, 1994). A distinct subset of the haemopoietin receptors form homodimers following ligand binding, including those for growth hormone (GH), prolactin and granulocyte colony-stimulating factor (G-CSF; Elberg *et al.*, 1990; Fukunaga *et al.*, 1990; Cunningham *et al.*, 1991; Fuh *et al.*, 1992; Hooper *et al.*, 1993). The erythropoietin (EPO) receptor appears also to be activated in this manner, as mutation of specific amino acids to cysteine residues

within the extracellular domain forces disulfide-linked homodimerization and results in constitutive, EPO-independent activation (Yoshimura *et al.*, 1990; Watowich *et al.*, 1992, 1994).

The crystal structure of the GH–GH receptor complex provides a model for haemopoietin receptor structure and subunit interaction. The haemopoietin domain consists of two subdomains, each composed of seven β-strands which form a sandwich of two antiparallel β-sheets (de Vos *et al.*, 1992). The GH–GH receptor complex, which contains a GH receptor homodimer bound by a single hormone molecule, is stabilized not only by ligand–receptor interactions but also through intermolecular interaction of amino acids located primarily within the same membrane-proximal domain of each receptor monomer. Significantly, the region of the EPO receptor that is sensitive to activation by cysteine substitution mutation aligns with the domain of the GH receptor most involved in dimer stabilization (Watowich *et al.*, 1994). These receptors therefore appear to share a structurally conserved dimer interface domain.

To explore the biochemical mechanisms and biological consequences of c-Mpl activation, we have generated constitutively active receptor mutants. We report here the identification of dimer interface homology regions within the c-Mpl receptor in an analogous position to those conserved between the GH and EPO receptors. Substitution of cysteine residues into this domain, designed to promote disulfide-bonded homodimerization, constitutively activated Mpl: factor-dependent cells expressing these mutants no longer required exogenous factors for growth and became tumorigenic. The results imply that the normal process of TPO-induced c-Mpl activation involves receptor homodimerization mediated by a dimer interface domain that is conserved among other haemopoietin receptor family members. Moreover, the tumorigenicity of cells expressing activated *mpl* mutants reveals that, like its viral counterpart, the cellular *mpl* gene is capable of contributing to oncogenesis.

Results

The amino acid sequences of the GH, EPO and c-Mpl receptor haemopoietin domains were compared using the conserved cysteine pairs, stretches of alternating hydrophobic and polar residues and the WSXWS motifs as major conserved landmarks and aligning the intervening residues for best homology. Figure 1A shows a localized region of this comparison around the dimer interface domain of the human GH receptor, which aligns with the region of the EPO receptor into which the introduction of cysteine residues causes constitutive dimerization and receptor activation (see also Watowich *et al.*, 1994). To determine whether activation of the c-Mpl receptor involves an analogous domain, residues in the dimer interface homology region of each of the two Mpl haemopoietin domains were mutated to cysteine. Codons 117 (*mpl*R117C) and 120 (*mpl*S120C) in the N-terminal haemopoietin domain, and 368 (*mpl*S368C) and 369 (*mpl*S369C) in the membrane-proximal domain were altered individually by site-directed mutagenesis to encode cysteine residues (Figure 1). Each mutant *mpl* cDNA, as well as the wild-type sequence, was inserted into the LXSN retroviral vector to allow expression in cells via viral infection (see Materials and methods).

Activity of c-mpl mutants

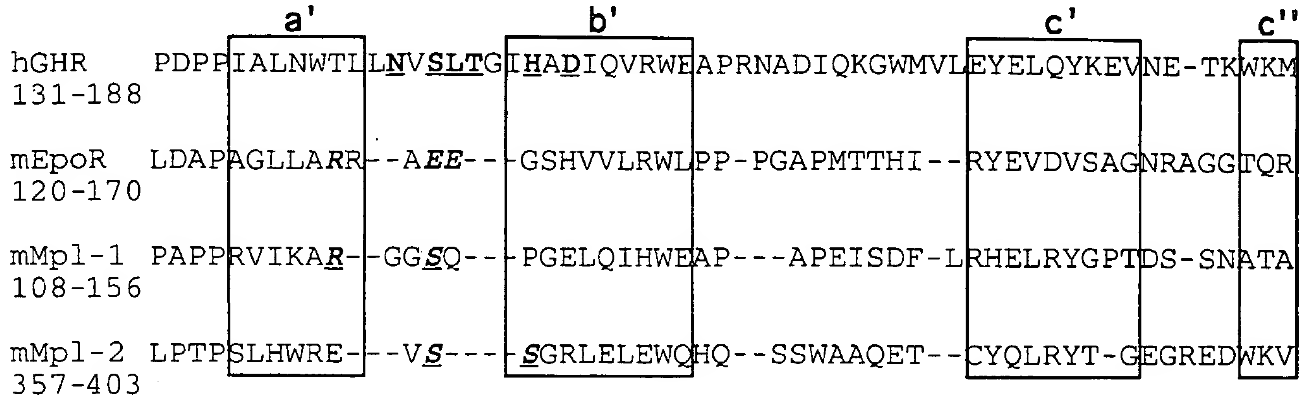
The activity of each *mpl* mutant was assessed by determining the capacity of the altered receptors to produce a proliferative signal in factor-dependent FDC-P1 and Ba/F3 cells. Parental (uninfected) cells were strictly dependent on exogenous IL-3 for survival and growth and did not produce colonies in agar in the absence of added factor (Figure 2). Upon infection with the LXSN virus or its wild-type (wt) or mutant *mpl* derivatives, each of which also carries the neo^R gene, the cells gained the capacity to grow in the cytotoxic drug G418, confirming that they had been productively infected (Figure 2). However, only cells infected with mutant *mpl* viruses acquired the ability to produce agar colonies in the absence of exogenous growth factors. Relatively high numbers of factor-independent colonies consistently arose from *mpl*S368C virus-infected FDC-P1 or Ba/F3 populations. Smaller numbers were observed in *mpl*S369C cultures, and only sporadic colonies arose from *mpl*R117C virus-infected cells. The *mpl*S120C mutant consistently failed to stimulate autonomous cell growth, as did the *mpl*wt receptor, as would be expected in the absence of ligand (Figure 2).

The ligand-independent activity of mutant Mpl receptors demonstrates that the substitution of cysteine residues for amino acids within a conserved dimer interface homology domain does indeed constitutively activate receptor function. To estimate more quantitatively the efficiency with which each mutant conferred factor independence, Ba/F3 cells were infected with the wt or mutant *mpl* viruses and selected for receptor expression in liquid cultures with a maximal concentration of TPO as the sole stimulus. Cells proliferated in all cultures, confirming that each of the *mpl* mutants was appropriately expressed and the proportion of autonomous cells in each population was then measured in agar cultures. As suggested in the initial experiments, the *mpl*S368C mutant most efficiently induced factor independence: 48% of cells selected to express sufficient *mpl*S368C to respond to TPO were capable of autonomous growth (Figure 3A). Only 7% of TPO-responsive Ba/F3-*mpl*S369C cells were factor-independent and no autonomous colonies arose from 10^3 receptor-expressing Ba/F3-*mpl*R117C, -*mpl*S120C or -*mpl*wt cells (Figure 3A). As expected, control cultures of uninfected or Ba/F3-LXSN cells failed to proliferate in TPO, as parental cells lack endogenous Mpl receptors. The dose–response of Ba/F3 cells expressing *mpl*R117C or *mpl*S120C to TPO was similar to that of Ba/F3-*mpl*wt cells in proliferation assays (Figure 3B). Thus, the inactivity or low efficiency of these mutants in stimulating autonomous growth do not appear to stem from poor expression or defective transport to the cell surface, nor from any intrinsic defect in signal transduction resulting from the cysteine substitution mutations.

Establishment of factor-independent clones

Factor-independent colonies from primary agar cultures of mutant *mpl* virus-infected cells were selected for expansion as clones in liquid culture. Essentially all colonies picked from FDC-P1 cultures expressing *mpl*R117C, *mpl*S368C or *mpl*S369C continued to prolifer-

A



B

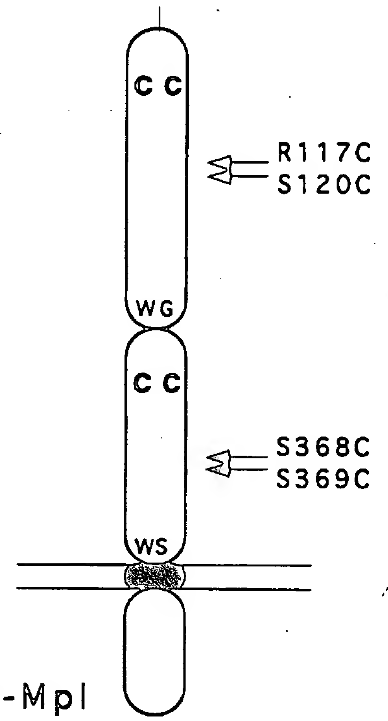


Fig. 1. **(A)** Amino acid sequence alignment of part of the haemopoietin receptor domains of the human growth hormone (hGHR), and murine EPO (mEPOR) and c-Mpl receptors. The region shown encompasses the GH receptor dimer interface domain (with residues involved in salt bridges or hydrogen bonds between receptor monomers underlined) and the domain of the EPO receptor where alteration of particular residues (italicized) to cysteines induces constitutive activation. The homologous region of both the N-terminal (mMpl-1) and membrane-proximal (mMpl-2) c-Mpl haemopoietin domains is also aligned, with residues targeted for mutation in this study both italicized and underlined. The region aligned from each receptor is indicated by amino acid number. The boxed sequences are regions of β-sheet structure (designated a', b', c'and c″) derived from the GH receptor crystal structure and predicted to exist in other haemopoietin receptors (Bazan, 1990; de Vos *et al.*, 1992). **(B)** Schematic representation of the c-Mpl receptor indicating the conserved cysteine residues (CC) and WSXWS motifs in each of the two haemopoietin domains and with the transmembrane region shaded. The positions of the amino acids changed to cysteines in this paper are indicated with arrows.

ate autonomously as did those from Ba/F3-*mpl*S368C and -*mpl*S369C cultures (Table I). Over several experiments, only one colony grew from Ba/F3-*mpl*R117C cells in the absence of added factors and it subsequently failed to expand when transferred into liquid culture. From both FDC-P1 and Ba/F3 cells, all colonies that expanded initially continued to proliferate upon further passage and were established as factor-independent cell lines.

To confirm the origin of these autonomous cell lines, genomic DNA was analysed by Southern blotting for retroviral integration. Proviruses containing the c-*mpl* cDNA were detected in all clones analysed, as shown for several examples in Figure 4. Using unique restriction endonucleases engineered into each mutant cDNA (see Materials and methods), the origin of each clone from the appropriate mutant *mpl* virus was also confirmed (data not shown). Interestingly, the number of proviral integrations was on average twice as high in FDC-P1- or Ba/F3-*mpl*S369C clones than in their *mpl*S368C counterparts (Figure 4 and Table I). The relative inefficiency of the S369C mutant in comparison with S368C in stimulating autonomous growth (Figure 3A) may demand higher expression levels resulting in selection of clones with multiple integrated proviruses (see Discussion).

Factor-independent proliferation of mplS368C cells is density independent

The growth properties of several clones of FDC-P1 and Ba/F3 cells expressing the constitutive *mpl*S368C mutant were analysed in detail. In addition to proliferating autonomously in liquid cultures and carrying integrated *mpl*S368C proviruses, Northern blot analysis was used to confirm that these clones also expressed the appropriate exogenous *mpl* transcripts (data not shown). The number of colonies formed in agar per cell plated (cloning efficiency) was 65–85% for FDC-P1-*mpl*S368C clones and 30–50% for Ba/F3-*mpl*S368C clones. The cloning efficiency of all clones was independent of the number of cells plated. Moreover, the addition of maximal concentrations of WEHI-3BD⁻-conditioned medium or TPO did not consistently influence the efficiency of colony formation (Figure 5). Media harvested from 5 ml cultures of each

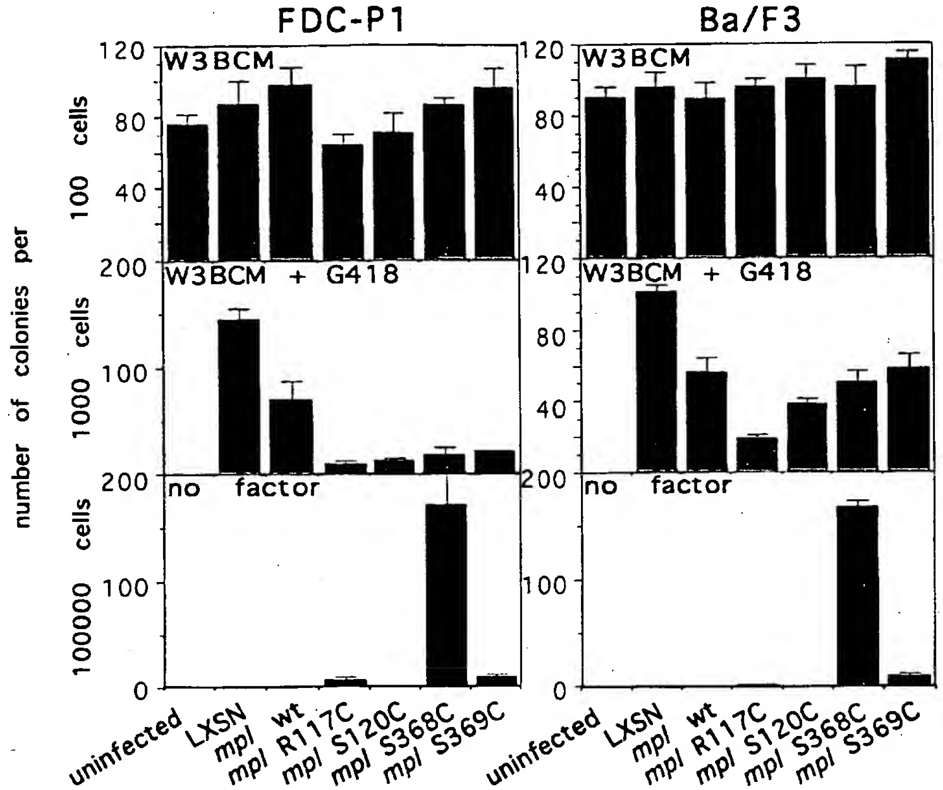


Fig. 2. Colony formation in agar cultures by uninfected FDC-P1 and Ba/F3 cell populations and following their infection with the LXSN virus or viruses expressing wild-type (wt) or cysteine-mutant (R117C, S120C, S368C, S369C) Mpl receptors. The bars show the mean number of colonies stimulated by WEHI3B-D⁻-conditioned medium (W3BCM), a source of IL-3, in the presence or absence of the cytotoxic drug G418 or in unstimulated (no factor) cultures. Error bars indicate the standard error of the mean of triplicate determinations.

of three FDC-P1- or Ba/F3-*mpl*S368C clones 24–48 h after seeding at 5×10^5 cells/ml were assayed for the production of growth factors. None of the clones secreted factors capable of stimulating Ba/F3 cells expressing wt Mpl receptors, even when the conditioned medium was concentrated 10-fold (data not shown). The density-independence of cell proliferation and the absence of detectable secreted stimuli suggest that the mutant MplS368C receptor induces factor independence in the absence of any apparent autocrine mechanism.

The role of disulfide-bonded dimerization in constitutive MplS368C activity

The substitution of cysteine residues into c-Mpl was designed to induce constitutive receptor activity through disulfide-linked homodimerization. To examine the structure of mutant Mpl receptors in factor-independent cells, Ba/F3 cell clones were derived that expressed normal (*mpl*wt-F) or S368C mutant (*mpl*S368C-F) Mpl receptors containing an epitope tag at their N-terminus. The epitope-tagged receptors exhibited identical properties to their untagged counterparts: Ba/F3-*mpl*wt-F cells responded to TPO but failed to grow in the absence of exogenous factors and Ba/F3-*mpl*S368C-F cells were autonomous (data not shown). Receptor protein was precipitated from cell lysates with an antibody directed against the epitope tag, separated in SDS–polyacrylamide gels and Western blotted with the same antibody (see Materials and methods). As shown in Figure 6A, both Ba/F3-*mpl*wt-F and factor-independent Ba/F3-*mpl*S368C-F cells abundantly expressed a protein that was absent in uninfected cells and was the size expected of the c-Mpl receptor (Skoda *et al.*, 1993). Consistent with the presence of disulfide-linked Mpl homodimers, significant levels of a protein species which migrated at a molecular weight approximately twice that of c-Mpl and that reduced to monomeric size upon treatment with 2-mercaptoethanol (2-ME), were precipitated from Ba/F3-*mpl*S368C cells (Figure 6A). A larger species that may have resulted from additional receptor aggregation was also observed in these cells under non-reducing conditions.

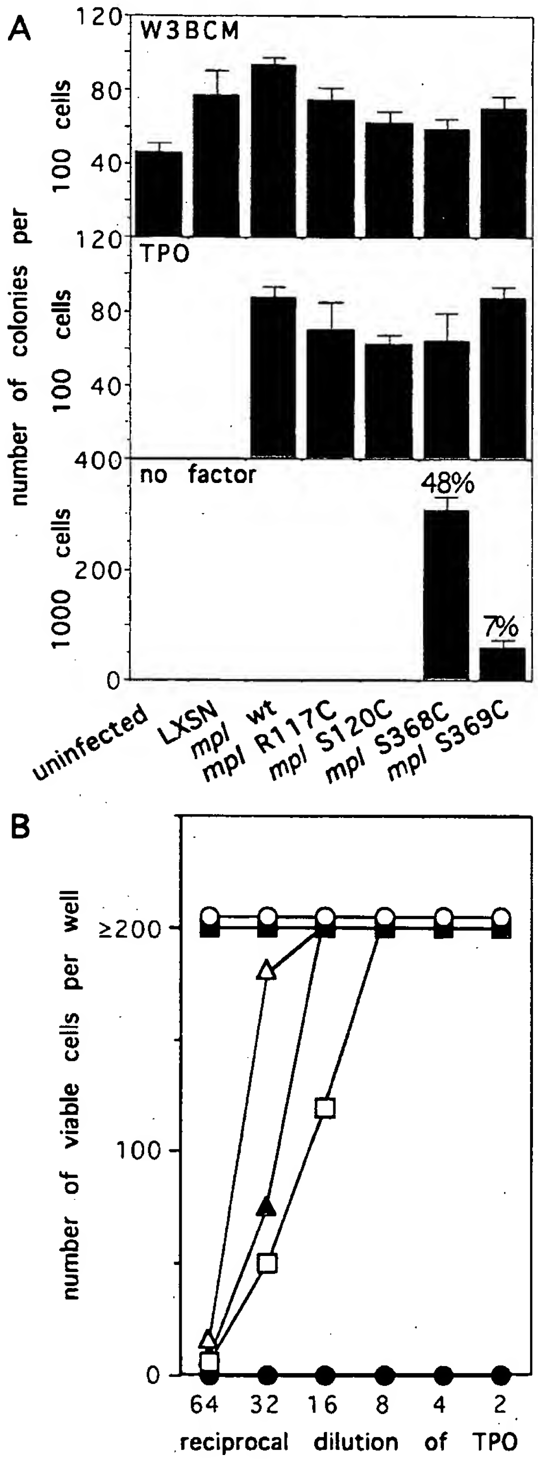
The proliferation of factor-independent cells was also assessed in the presence of chemical reducing agents. At appropriate concentrations of 2-ME, the reduced form of glutathione or α-monothioglycerol (α-MTG), factor-independent growth of FDC-P1-*mpl*S368C cells was inhibited (Figure 6B), consistent with dependence on disulfide bond formation for receptor activity. In the presence of TPO, when the cells no longer depend on constitutive receptor activity, proliferation was unaffected by the presence of reducing agents (Figure 6B). Similar results were obtained with Ba/F3-*mpl*S368C cells (data not shown). With the protein studies above, these data strongly imply that MplS368C receptors form disulfide-linked dimers and that this dimerization is critical for constitutive receptor activity.

Cells expressing mplS368C are tumorigenic

When 10^6 cells of each of three FDC-P1-*mpl*S368C clones were injected subcutaneously into syngeneic DBA/2 mice, tumours became apparent at the site of injection within 2–4 weeks. No mice injected with parental factor-dependent

FDC-P1 cells developed tumours within a 90 day observation period (Table II). In addition to a subcutaneous tumour at the site of injection, FDC-P1-*mpl*S368C cells also induced considerable splenomegaly and occasional enlargement of local lymph nodes in transplanted animals. Several mice also exhibited extensive intra-abdominal tumours and ascites fluid. Ba/F3-*mpl*S368C cells exhibited a somewhat weaker tumorigenic phenotype. Tumours arose only in irradiated (500 rad) recipient mice and not all injected mice succumbed. Nevertheless, the mutant MplS368C receptor clearly predisposed Ba/F3 cells to tumorigenicity, as uninfected cells or those expressing normal Mpl receptors were not tumorigenic (Table II). The majority of tumours arising in Ba/F3-*mpl*S368C-transplanted mice developed at the injection site without obvious splenomegaly or involvement of other organs.

A proviral integration pattern identical to that of the injected cells was detected in DNA from subcutaneous tumour or ascites samples taken from each tumour-bearing mouse (data not shown), confirming that the tumours originated from the appropriate autonomous FDC-P1- and Ba/F3-*mpl*S368C clones. Thus, constitutive receptor activation induced by mutation reveals that the cellular *mpl* gene, like its v-*mpl* counterpart, can contribute to tumorigenesis (see Discussion).



Discussion

An homologous domain within two members of the haemopoietin receptor superfamily has been implicated in mediating dimerization during receptor activation. Specific residues in the haemopoietin homology domain (residing within or adjacent to the loop between the a′ and b′ β-strands) of GH receptor monomers participate in hydrogen bonding and salt bridges to help stabilize ligand-induced receptor homodimers (Figure 1 and de Vos *et al.*, 1992).

Table I. Establishment of factor-independent cell clones and analysis of proviral integration

Cells	Frequency of expansion of agar colonies in liquid culture[a]	No. of proviral integrations per clone[b]
FDC-P1-*mpl* R117C	13/13	4.3 ± 2.5 (n = 6)
FDC-P1-*mpl* S368C	12/12	1.6 ± 0.5 (n = 9)
FDC-P1-*mpl* S369C	15/16	3.2 ± 2.1 (n = 6)
Ba/F3-*mpl* R117C	0/1	–
Ba/F3-*mpl* S368C	12/12	4.2 ± 1.5 (n = 6)
Ba/F3-*mpl* S369C	12/12	7.8 ± 3.1 (n = 6)

[a]Colonies growing in the absence of exogenous growth factors in primary agar cultures of infected cells were picked into DMEM + 10% FCS. The number of colonies that expanded into an established line is expressed as a ratio of the number of colonies transferred.
[b]Mean ± standard error of the number of independent proviral integration sites assessed using both c-*mpl* cDNA and *neo* probes in Southern blot analysis of established clones of factor-independent cells.
n denotes the number of clones analysed.

Fig. 3. (**A**) Efficiency of induction of factor independence by *mpl* mutants. Colony formation in agar by uninfected Ba/F3, Ba/F3-LXSN and Ba/F3 cells expressing wild-type (wt) or mutant (R117C, S120C, S368C, S369C) Mpl receptors in response to WEHI3B-D$^-$-conditioned medium (W3BCM), TPO or in the absence of exogenous factors (no factor) is shown. Cells expressing Mpl receptors were pre-selected for 14 days in liquid cultures with TPO. Ba/F3-LXSN cells were selected in W3BCM plus G418. The mean and standard error of triplicate cultures are graphed. 48% of the number of Ba/F3-*mpl*S368C cells and 7% of Ba/F3-*mpl*S369C cells that grew in TPO were autonomous. No autonomous cells were detected in any of the other cell populations. (**B**) Dose–response of infected Ba/F3 cell populations to TPO. Two-hundred uninfected Ba/F3 cells (●) or Ba/F3 cells expressing wild-type (▲), R117C (△), S120C (□), S368C (○) or S369C (■) *mpl* mutants were placed in microwells with serially diluted concentrations of TPO. The mean of duplicate cell counts after 48 h is shown. Infected cell populations were derived as in (A). The Ba/F3-*mpl*S368C and Ba/F3-*mpl*S369C populations contain factor-independent cells and are therefore maximally stimulated at all TPO concentrations.

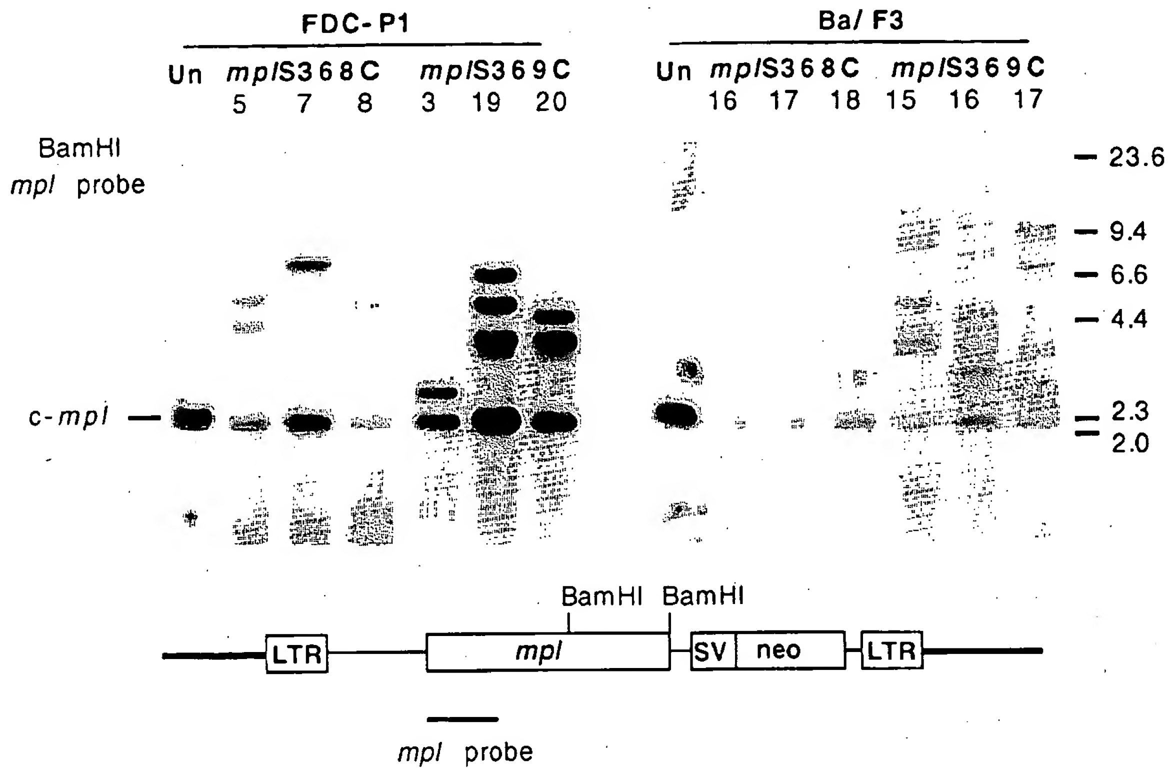


Fig. 4. Proviral integration in factor-independent cell clones. Southern blots of genomic DNA from uninfected (Un) and individual numbered clones of established FDC-P1- and Ba/F3-*mpl*S368C and *mpl*S369C cells. The DNA was digested with *Bam*HI, which cuts within the *mpl* proviruses such that individual proviral integrants will yield uniquely sized fragments with the *mpl* probe indicated. The position of migration of the endogenous c-*mpl* allele and molecular weight size markers in kbp are also shown.

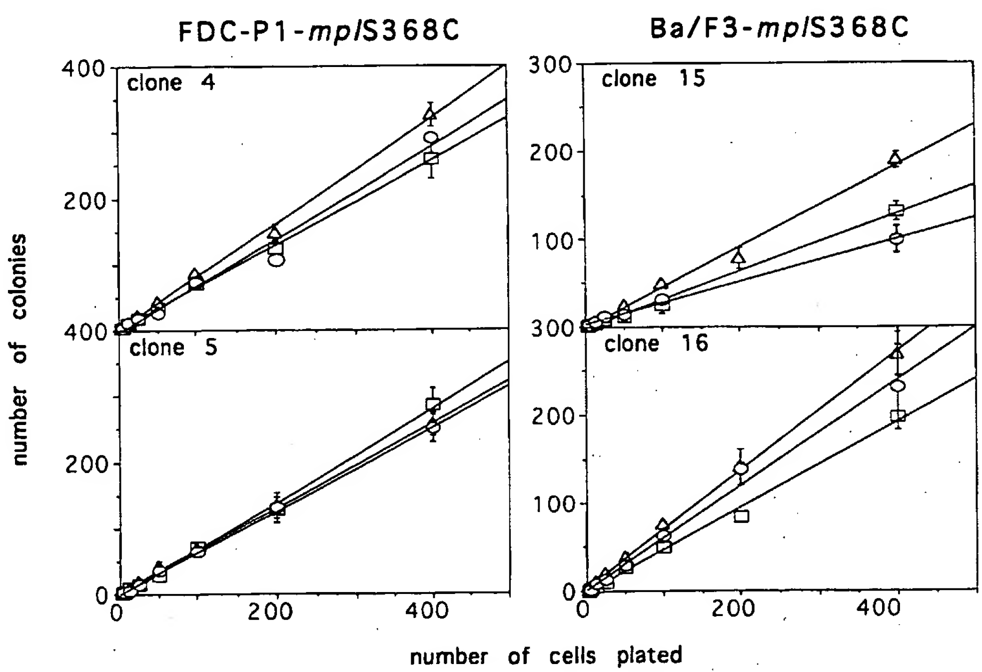


Fig. 5. Density-independent proliferation of factor-independent cells. Individual clones of FDC-P1- and Ba/F3-*mpl*S368C cells were plated at various densities in agar cultures with saturating doses of WEHI3B-D⁻-conditioned medium (△), TPO (○) or in the absence of added factors (□). The mean and standard error of triplicate determinations is shown.

The analogous region of the EPO receptor also seems to mediate dimerization, as the substitution of cysteine residues for particular amino acids in this domain results in ligand-independent receptor activation through disulfide-bonded homodimerization (Yoshimura *et al.*, 1990; Watowich *et al.*, 1992, 1994). The results presented here demonstrate that the c-Mpl receptor also can be activated constitutively by the introduction of cysteine residues into

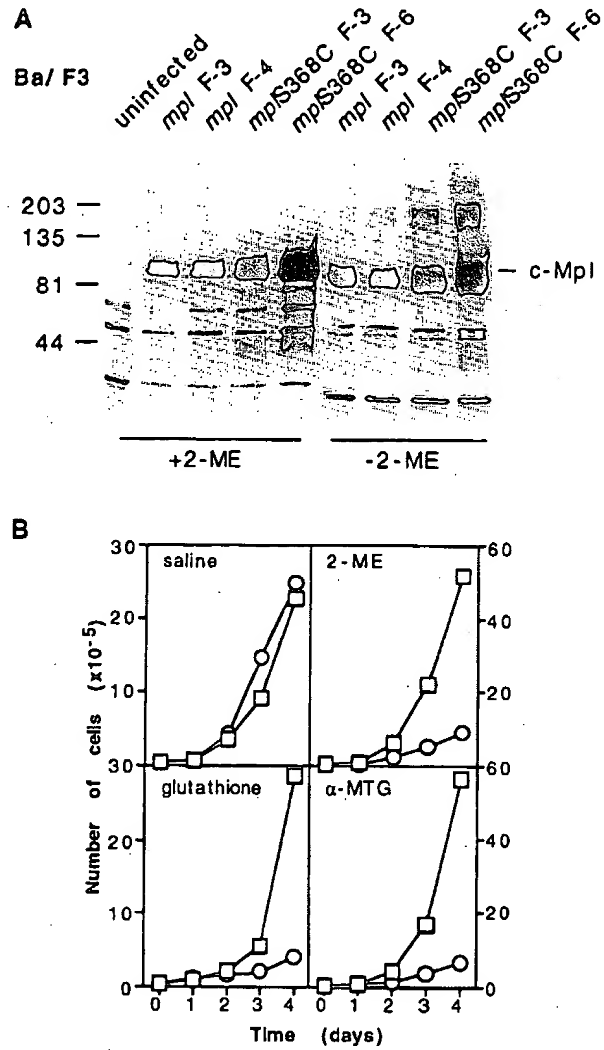


Fig. 6. (**A**) c-Mpl protein in Ba/F3 cells expressing FLAG epitope-tagged wt (*mpl* F-3, F-4) and S368C mutant (*mpl*S368C F-3, F-6) receptors. Proteins precipitated with the anti-FLAG M2 antibody were left untreated or incubated with 2% 2-ME, separated by SDS–PAGE and blotted to nitrocellulose membranes. The Western blot using the same M2 antibody is shown. The predicted size of the c-Mpl protein (monomeric) is indicated, as are molecular weight standards in kDa. (**B**) Inhibition of factor-independent cell proliferation by chemical reducing agents. FDC-P1-*mpl*S368C cells were plated in liquid cultures containing 2-ME (1.6×10^{-4} M), glutathione (reduced form; 1.2×10^{-2} M), α-MTG (1.0×10^{-2} M) or in control cultures (saline). Proliferation in the presence (□) or absence (○) of TPO was monitored daily by counting viable cells in each culture.

homologous dimer interface regions identified within its haemopoietin domains. Factor-dependent FDC-P1 or Ba/F3 cells expressing these activated mutant Mpl receptors no longer required exogenous factors for their survival and growth (Figure 2). Their autonomous proliferation was not dependent on cell density (Figure 5) and no secreted factors that stimulate cell growth could be detected, consistent with a constitutive proliferative signal emanating directly from the mutant receptor. Biochemical studies in cells expressing the *mpl*S368C mutant revealed a Mpl protein species of dimeric size that reduced to monomers upon treatment with 2-ME. Moreover, reducing agents inhibited the factor-independent growth of FDC-P1-*mpl*S368C cells (Figure 6). Together, these data strongly support the model that the introduction of cysteine residues into a dimer interface homology domain of c-Mpl induces constitutive receptor activity through disulfide-bonded homodimerization.

Previous studies with G-CSF receptor–c-Mpl chimeras (Vigon *et al.*, 1993b; Baumann *et al.*, 1994) and a variant of the constitutively active v-Mpl oncoprotein (Courtois *et al.*, 1995) have demonstrated that homodimerization of the Mpl intracellular domain induces receptor activity. Our studies here confirm these observations and extend them by providing strong evidence that receptor homodimerization is involved in the normal process of ligand-mediated Mpl activation. Our results suggest that the GH receptor dimer interface domain is conserved functionally in the loop region between the a′ and b′ β-strands of the haemopoietin domain, not only in the EPO receptor but also within c-Mpl. Given the structural similarity of haemopoietin receptor family members, the analogous regions of other haemopoietin receptors may also mediate the subunit associations that characterize their activation. Indeed, in mutation studies of the G-CSF receptor, the N-terminal half of the haemopoietin domain is sufficient to bind G-CSF at low affinity, but for high affinity binding, which correlates with homodimer formation (Fukunaga *et al.*, 1990), the full domain must be present (Fukunaga *et al.*, 1991). Several IL-6 receptor α-chain mutants have been described that can bind IL-6 but are unable subsequently to interact with gp130 for signal transduction. However, in this receptor, most of the mutations cluster in the region of the predicted e′ and f′ β-strands of the haemopoietin domain (Yawata *et al.*, 1993). Intriguingly, although the majority of residues contributing to stabilization of GH receptor dimers reside in the a′–b′ loop region, two are also located in the e′ β-strand, which lies adjacent (de Vos *et al.*, 1992). If the mechanism of subunit interaction is indeed similar among the haemopoietin receptors, a conserved subunit interface may thus be composed of two domains, although their relative contribution may vary between receptors.

The emergence of key domains in haemopoietin receptor subunit interactions has important implications for receptor function. Two independent cases of Laron syndrome, a familial resistance to GH, have arisen through mutations in the dimer interface domain of the GH receptor that prevent ligand-induced homodimerization (Duquesnoy *et al.*, 1994). Thus, in addition to being potential sites for mutational activation, dimer interface domains such as that identified here in c-Mpl, may also provide a target for approaches designed to inhibit haemopoietin receptor function. Our studies also highlight parallels between activation of haemopoietin receptors and receptors of the structurally unrelated tyrosine kinase family. Dimer formation is associated with activation of many tyrosine kinase receptors, and mutations that stabilize dimerization have been found to stimulate kinase activity and transforming capacity (Weiner *et al.*, 1989; Sorokin *et al.*, 1994).

c-Mpl belongs to the minority of haemopoietin receptors that have two haemopoietin homology domains within their extracellular sequence. Substitution of cysteine residues into either of these domains yielded receptors with constitutive activity. However, of the two receptors derived by mutations within the N-terminal domain, only MplR117C displayed very weak TPO-independent activity.

Table II. Tumorigenicity of factor-independent *mpl*S368C cells

Cells injected[a]	Mouse strain (irradiation dose)	Mice developing tumours/No. injected	Tumor latency (days)
FDC-P1	DBA/2	0/3	
FDC-P1-*mpl*S368C.4	DBA/2	3/3	20, 20, 26
FDC-P1-*mpl*S368C.5	DBA/2	3/3	16, 17, 27
FDC-P1-*mpl*S368C.12	DBA/2	3/3	28, 22, 23
Ba/F3	Balb/c	0/3	
	Balb/c (500 rad)	0/3	
Ba/F3-*mpl*wt	Balb/c (500 rad)	0/3	
Ba/F3-*mpl*S368C.15	Balb/c	0/3	
	Balb/c (500 rad)	2/3	34, 34
Ba/F3-*mpl*S368C.16	Balb/c	0/3	
	Balb/c (500 rad)	1/3	40
Ba/F3-*mpl*S368C.18	Balb/c	0/3	
	Balb/c (500 rad)	1/3	70

[a]10^6 cells were injected subcutaneously into the flank of each mouse.

This was not due to poor expression, deficiencies in transport to the cell surface or defective signal transduction, as cells expressing MplR117C or MplS120C could respond to TPO as efficiently as cells expressing Mplwt (Figure 3). In contrast, both membrane-proximal domain mutants (MplS368C and S369C) displayed constitutive activity (Figures 2 and 3). With the caveat that the precise residues within the N-terminal domain may not have been targeted for optimal activation in our study, these results may indicate a more significant contribution to Mpl activation by the membrane-proximal dimer interface homology domain. Even within the membrane-proximal domain, the two independent Mpl mutants appeared to differ in potency. Of all Ba/F3 cells expressing sufficient levels of the *mpl*S368C mutant to respond to TPO, around half were autonomous. In contrast, only 7% of TPO-responsive Ba/F3-*mpl*S369C cells were factor independent (Figure 3A). Similarly, only a subset of residues in the EPO receptor dimer interface domain induce constitutive activity when substituted with cysteines (Watowich *et al.*, 1992). In the homodimerization model, it seems likely that, at different positions within the dimer interface domain, the orientation of substituted cysteine residues will influence critically the efficiency of disulfide bond formation between adjacent mutant receptors. Higher efficiency of bond formation between *mpl*S368C monomers than those of *mpl*S369C could explain their differential capacities to induce factor-independent colony formation. If a threshold level of homodimer formation is required to stimulate cellular proliferation, higher numbers of receptors may be required in autonomous *mpl*S369C cells than in equivalent *mpl*S368C cells. We have observed a comparatively higher average number of *mpl*S369C retroviral integration sites (Figure 4 and Table I), which may indicate selection for elevated expression levels.

In addition to becoming factor independent, FDC-P1 and Ba/F3 cells expressing the *mpl*S368C mutant also induced tumours in transplanted mice. FDC-P1-*mpl*S368C cells were highly tumorigenic, with all injected mice from three independent clones succumbing within 2–4 weeks. Ba/F3-*mpl*S368C cells were less potent and also required prior irradiation of the recipient mice (Table II). Although v-*mpl*, albeit a grossly mutated receptor form, is highly tumorigenic (Wendling *et al.*, 1986), to date the cellular *mpl* gene has not been found to be reproducibly over-expressed in human myeloid leukaemias (Vigon *et al.*, 1993a). Our results extend the studies of v-*mpl* to show that deletion of extracellular receptor domains and/or the presence of viral *env* sequences are not mandatory for constitutive receptor activity, and further demonstrate that, when activated by point mutation, the cellular *mpl* gene is tumorigenic in established haemopoietic cell lines. It is feasible, therefore, that subtle alterations to the c-*mpl* gene which influence receptor activity may be found to contribute to the development of leukaemia.

Materials and methods

In vitro mutagenesis

Mutations were engineered into the murine *mpl* cDNA using a modification of the site-directed mutagenesis technique of Kunkel (1985). Briefly, a phagemid containing the full-length c-*mpl2* cDNA (Alexander and Dunn, 1995) in BluescriptKS(+) was transformed into *Escherichia coli* CJ236 (*dut*$^-$, *ung*$^-$) cells from which single-stranded, dUTP-containing template DNA was prepared. Each mutagenesis oligonucleotide (250 ng) was annealed to the template DNA (500 ng) and then incubated with T7 DNA polymerase (2 U; Sequenase, USB, Cleveland, OH) and T4 DNA ligase (1 U; Promega, Madison, WI) in Sequenase buffer supplemented with 650 μM dNTP, 500 μM ATP and 1.5 mM dithiothreitol (DTT) to synthesize and ligate the mutated phagemid strand. This reaction product was transformed into *E.coli* NM522 (*dut*$^+$, *ung*$^+$) cells to select against the dUTP$^+$ parental strand. Individual clones were initially screened for the presence of novel restriction endonuclease sites (see below), and then sequenced to confirm the introduction of the desired mutation and to exclude secondary mutations. The mutagenesis oligonucleotides, designed to alter specific codons in c-*mpl* to those for cysteine, and also incorporate silent nucleotide changes to introduce novel restriction endonuclease sites (*Stu*I for *mpl*R117C, *Sma*I for *mpl*S120C, *Pst*I for *mpl*S368C and *Aat*II for *mpl*S369C), are antisense to the c-*mpl* coding strand and have the following sequences: *mpl*R117C 5′-GGCTCCCACCGCAGGCCTTGATG-3′; *mpl*S120C 5′-GAAGTTC-CCCGGGTTGGCACCCACCCCTG-3′; *mpl*S368C 5′-CTCCAGCC-TTCCACTGCAGACCTCCCTCCAGTG-3′; *mpl*S369C 5′-CCACTC-CAACTCCAGACGTCCGCATGAGACCTCCCTCCA-3′. The codon numbers 117, 120, 368 and 369 represent amino acid positions in the mature Mpl protein (Skoda *et al.*, 1993), while underlined nucleotides indicate residues altered from the wild-type sequence. Each *mpl* mutant, as well as the wild-type cDNA, was incorporated into the LXSN retroviral vector (Miller and Rosman, 1989) in which the receptor is expressed from the 5′ long terminal repeat. The vector also includes an internal *neo*^{R} gene driven by an SV40 promoter allowing selection for infected cells with geneticin (G418 sulfate, Gibco, NY). Stable cell lines releasing infectious, helper-free virus were obtained from these receptor constructs via transfection into Ψ-2 packaging cells (Mann *et al.*, 1983) and selection for G418 resistance.

Cell lines and retroviral infections

The FDC-P1 (Dexter *et al.*, 1980) and Ba/F3 (Palacios and Steinmetz, 1985) haemopoietic cell lines are strictly dependent on exogenous factors, including IL-3, for survival and growth and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10% WEHI-3BD$^-$-conditioned medium (Metcalf, 1984). Cells were infected with retroviruses expressing wild-type or mutant Mpl receptors by exposure to viral supernatants for 2 h or by co-cultivation with Ψ-2 virus-producing cells for 48 h in growth medium supplemented with 4 μg/ml polybrene (hexadimethrine bromide, Sigma, St Louis, MO).

Agar cultures

Infected cells or factor-independent clones were washed by centrifugation in phosphate-buffered saline (PBS), resuspended at the desired concentration in DMEM containing 10% FCS and 0.3% agar (Metcalf, 1984) and plated as 1 ml cultures in 35 mm Petri dishes. Cultures were stimulated with WEHI-3BD$^-$-conditioned medium (10%, as a source of IL-3), the medium conditioned by COS cells expressing recombinant human TPO, kindly provided by Drs T.Willson and M.Rossner (The Walter and Eliza Hall Institute of Medical Research), or with normal saline. Where added, G418 was used at 1.2 mg/ml. Colony formation was scored after 7 days incubation at 37°C in a fully humidified atmosphere of 10% CO_2 in air.

Microwell assays

The response of cell lines to TPO was measured in Lux 60 microwell HLA plates (Nunc Inc., Roskilde, Denmark). Cells were washed in DMEM containing 10% FCS and 10 μl aliquots containing 200 cells in the same medium were placed in each microwell with 5 μl of serially diluted conditioned medium containing TPO. The numbers of viable cells in each well were counted after incubation for 2 days at 37°C in a fully humidified atmosphere of 10% CO_2 in air. Serially diluted media conditioned by factor-independent clones were assayed in identical fashion for growth stimulatory activity on Ba/F3 cells engineered to exogenously express the normal Mpl receptor.

Nucleic acid analyses

For genomic DNA extraction, cells were washed twice in PBS and resuspended in 50 mM Tris–HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS, 500 μg/ml proteinase K at pH 8. After incubation at 50°C for 16 h, the lysate was extracted twice with a 25:25:1 mixture of phenol, chloroform and iso-amyl alcohol (PCIAA) and the genomic DNA precipitated with isopropanol in the presence of 300 mM sodium acetate. For Southern blots, 15 μg of DNA were digested to completion with the desired restriction endonuclease and then electrophoresed through 0.7% agarose gels. The DNA was transferred to nylon membrane (Genescreen Plus, NEN, Boston) by soaking the gel sequentially in 0.25 M HCl and then 0.5 M NaOH, 1.5 M NaCl for 20 min each before blotting with the same alkali buffer. After transfer, the membranes were neutralized in 40 mM Na_2HPO_4, pH 7.2.

RNA was purified by dissolving cells in 4 M guanidine thiocyanate, 25 mM sodium citrate, 1% 2-ME, 0.5% lauryl sarcosine, then extracting the lysate with PCIAA and precipitating the RNA from the aqueous phase with isopropanol. The polyadenylated RNA fraction was purified by oligo(dT) chromatography. Briefly, total cellular RNA was dissolved in 0.5 M NaCl, 10 mM EDTA, 20 mM Tris–HCl, pH 7.5 and incubated with 50–100 mg oligo(dT)-cellulose (Boehringer, Mannheim) at room temperature for 1–2 h. The cellulose was washed thoroughly in 0.4 M NaCl, 10 mM EDTA, 0.2% SDS, 20 mM Tris–HCl, pH 7.5 and then 0.1 M NaCl, 10 mM EDTA, 0.2% SDS, 10 mM Tris–HCl, pH 7.5 before the poly(A)$^+$ RNA was eluted in 1 mM EDTA, 0.2% SDS, 1 mM Tris–HCl, pH 7.5 at 60°C. Northern blots were performed by electrophoresis of poly(A)$^+$ RNA (5 μg) through 1.2% agarose gels containing 6% formaldehyde followed by blotting to nylon membranes (Genescreen Plus, NEN, Boston) in 10× SSC (1.5 M NaCl, 150 mM sodium citrate, pH 7.0).

Northern and Southern blot membranes were hybridized with cDNA probes labelled with [α-^{32}P]dATP using a random decanucleotide priming system (Bresatec, Adelaide, South Australia) and separated from unincorporated radioactivity by ethanol precipitation from 2.5 M ammonium acetate. After hybridization in 0.5 M Na_2HPO_4, 7% SDS, 1 mM EDTA, pH 7.2 at 65°C for 16 h, membranes were washed twice in 40 mM Na_2HPO_4, 1% SDS, pH 7.2 for 30 min at 65°C and exposed to autoradiographic film. The *mpl* probe used was a 0.23 kb fragment of c-*mpl* cDNA extending from the initiation ATG codon to the *Sph*I site downstream.

Protein analyses

Ba/F3 cells (10^6) expressing normal or mutant Mpl receptors engineered with a FLAG (Asp–Tyr–Lys–Asp–Asp–Asp–Lys) peptide epitope at the N-terminus of the mature protein were lysed in 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, for 5 min on ice in the presence of protease inhibitors. Lysates were clarified by centrifugation and Mpl receptors precipitated with anti-FLAG M2 affinity gel (Eastman Kodak, New Haven, CT) for 2 h at 4°C. Immunoprecipitates were washed twice in lysis buffer, half of each sample was incubated for 5 min with 2-ME (2%) and, after boiling in 1% SDS, 10% glycerol, 80 mM Tris-HCl, pH 6.8, were separated by electrophoresis in SDS–polyacrylamide gels and electro-transferred to nitrocellulose membranes (Amersham, UK). Filters were incubated with the same M2 antibody and protein visualized using the Amersham (UK) ECL system.

Transplantation

Factor-independent FDC-P1 and Ba/F3 clones were assayed for tumorigenicity in 8- to 20-week-old syngeneic DBA-2 and Balb/c mice respectively. Mice receiving Ba/F3 cell clones were divided into two groups, one of which was irradiated with 500 rad whole body irradiation prior to injection. 10^6 cells were washed twice in DMEM and injected subcutaneously into the flank region of each of three mice per assay.

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